ENDOMETRIAL LEUCOCYTES AND THEIR RELATION TO FERTILITY

DR. JANE A. STEWART

MD THESIS
THE UNIVERSITY OF EDINBURGH
2000

This thesis is dedicated to my husband Graham, whose patience and support has been so important to its completion, and to our children Kirstin and Lucy.

ABSTRACT

The aim of this study was to establish further knowledge of the endometrial cyclical immune response and its controls and to relate this to previous pregnancy and in particular unexplained subfertility. In addition the possibility of examining the direct effect of early embryo products on individual endometrial leucocyte populations *in vitro* was explored.

The clinical aspects of this work were performed in the Centre for Reproductive Medicine, Royal Victoria Infirmary, Newcastle-upon-Tyne and laboratory work was performed in the Departments of Pathology and Immunology.

For the majority of the study subjects were recruited into one of four groups; parous fertile, parous infertile, nulliparous, presumed fertile and nulliparous infertile. Timed endometrial biopsies were obtained at 7 and 13 days following the urinary luteinising hormone surge in separate cycles from these subjects. Further studies were performed using endometrial biopsies obtained from hysterectomy specimens and decidua from first trimester terminations of pregnancy.

Endometrial leucocyte populations, their distributions and characteristics and their endometrial milieu were examined primarily using double and single labelling immunohistochemical techniques.

This work has shown that there are significant differences in the leucocyte populations of infertile compared with fertile endometrium. In particular CD56-positive endometrial granulated lymphocytes are present in greater numbers in the early secretory phase in infertile endometrium and undergo a smaller increase in numbers as the cycle proceeds. In addition a different balance of T-helper and cytotoxic T cells in both subfertile and fertile endometrium at LH+7 compared with LH+13 has been seen. A significant difference in endometrial leucocyte populations resulting from parity alone has been excluded such that the need to examine parous and nulliparous endometrium separately when investigating endometrial leucocyte populations appears to be negated, thus validating the results of some previous authors.

It has also been shown in this study, that there can be no direct ovarian steroid effect on endometrial leucocytes as they fail to express either oestrogen or progesterone receptors. It has been shown however, that endometrial steroid hormone receptor expression is altered by parity and fertility status, in particular surface epithelial progesterone receptor expression. Previously reported differences in some adhesion molecule distributions between fertile and subfertile endometrium have also been confirmed.

Further studies have shown that it is possible to isolate T cells from Pipelle samples of endometrium in great enough numbers and purity to examine their functions *in vitro* without prior cloning. In addition modified lymphocyte proliferation assays have been used to examine embryo product influences on leucocyte responses. It would be possible therefore to study the effects of individual embryo culture supernatants on isolated endometrial leucocyte populations. This opens the way to further explore the gene product functions of an individual blastocyst.

Thus these studies have contributed to the understanding of endometrial function in relation to fertility and contributed to the promotion of further experimentation in this field.

TABLE OF CONTENTS

TABLE OF FIGURES

TABLE OF TABLES

CHAPTER 1

INTRODUCTION

INTRODUCTION

A couple is considered to be subfertile if they have failed to conceive after at least two years of regular sexual intercourse. It is a problem affecting around 1 in 7 couples. In the UK it has been estimated (Fig. 1.1) that about 20% of subfertility is primarily tubal in origin (blocked or damaged Fallopian tubes), 30% related to ovulation failure (both female causes) and about 25% of fertility problems are mainly male factor in origin. It is of interest that in around 38% of couples more than one disorder is detectable, often a combination of male and female problems, whilst in approximately 25% of cases, routine examination and investigation of a couple fails to diagnose a cause (Hull 1992).

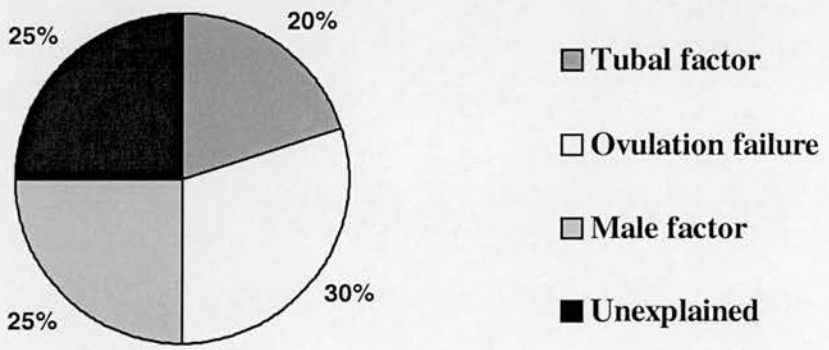


Figure 1.1. *Primary causes of infertility.*

Fertility Investigation

Standard fertility investigations include a thorough history from and examination of both partners and, depending on specific features of these, a battery of endocrine tests. In addition laparoscopy and dye insufflation or other tubal patency tests are performed and ovulation is confirmed in the female partner, usually by estimation of

secretory phase serum progesterone concentration. Semen analysis is performed for the male partner (RCOG 1992, 1998).

Unexplained Subfertility

Unexplained subfertility is generally defined as a failure to conceive over 2 years with regular sexual intercourse in the face of normal fertility investigations. It is unlikely however, that these couples are all identically affected; for example, in some normal couples chance alone may have been responsible for their failure to conceive, although 95% of "normal" couples will have conceived within two years of regular intercourse (Fig. 1.2) (Hull et al. 1985).

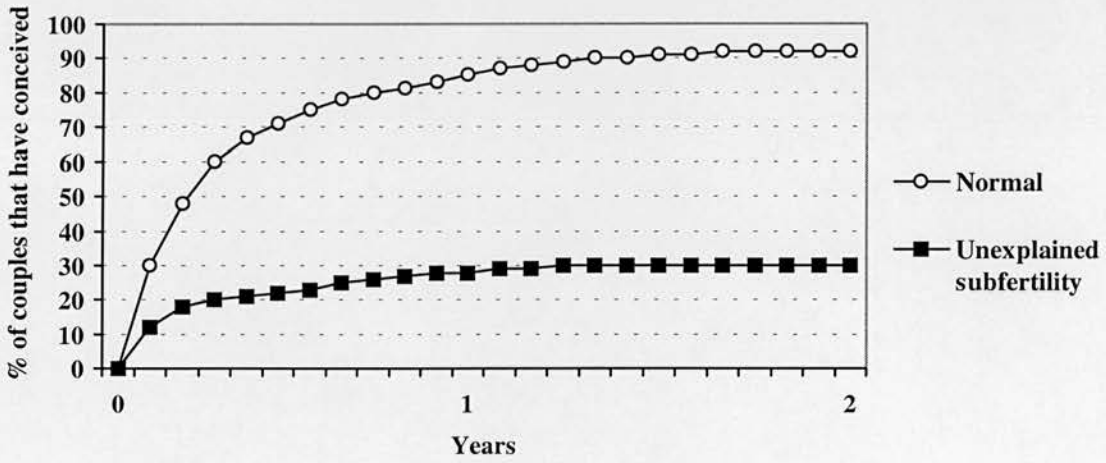


Figure.1.2. *Cumulative conception rates in normal fertile couples and couples with unexplained subfertility of greater than 5 years duration (Hull et al. 1985).*

Subtle combinations of sperm abnormalities or cervical mucus interactions may play a role, whilst lifestyle, psychological and emotional factors may all play a part to some degree in the aetiology of subfertility. Specific areas which are not amenable

to standard investigations but which would be important candidates for the site of abnormality in some cases of unexplained subfertility include; egg and sperm function; tubal function as opposed to anatomy; the tubal environment acting as the milieu for fertilisation; early embryo development and, most importantly, the endometrium and its interactions with that embryo.

The endometrium must be implicated in at least some cases of otherwise unexplained subfertility because of its relations to the embryo and its ability to support implantation and trophoblast invasion.

Fertility Treatment

The main strategies for the management of fertility problems are correction or bypassing of the problem. Tubal surgery and ovulation induction correct some of the underlying problems, whilst abnormalities of sperm structure or function may be by-passed by means of specialised *in vitro* fertilisation (IVF) techniques such as intracytoplasmic sperm injection (ICSI). By definition, as no specific pathology is identifiable in couples with unexplained subfertility, directed corrective measures are impossible. The treatment of choice therefore is to again by-pass any potential problems and, whilst some units will perform intrauterine insemination with or without ovulation induction, the "bottom-line" for management of this condition is often assisted reproductive techniques (ART), namely *in vitro* fertilisation (IVF) or gamete intra-fallopian transfer (GIFT). These can give a pregnancy success rate of approximately 15-40% per cycle undertaken (Hull 1992) but as such, treatment is relatively unsuccessful, with many couples failing to conceive each cycle.

Although it is to be hoped that no pregnancy is undertaken lightly, the decision to proceed to ART is a major one, which if successful may be amply rewarded. There is a significant cost however, not only in financial terms but also emotionally and socially to the couple undergoing ART particularly when unsuccessful. There is also a not insignificant physical risk to otherwise healthy individuals. Major complications include ovarian hyper-stimulation syndrome (OHSS), thromboembolism and multiple pregnancy with its associated risks. It would thus be of benefit to all, providers and patients alike if simpler, more straightforward measures could be taken to correct at least some of problems currently labelled as unexplained. Potential endometrial problems are not necessarily addressed by these treatment strategies, although they may be modified by hormone regimes. Attention needs to be paid to this aspect because endometrial factors causing subfertility may well also have a bearing on the success of any of the above interventions.

There are as yet no clear diagnostic features relating to the endometrium in unexplained subfertility and no proven therapeutic remedies in relation to endometrial dysfunction. Thus although some investigators will perform luteal phase endometrial biopsy, it is difficult at present to justify this procedure as anything other than a research tool (Balasch et al. 1992, RCOG 1998).

The Endometrium

The endometrium lining the uterine cavity is a dynamic tissue throughout the reproductive years. In the human, monthly menstrual cycles involve the shedding and rebuilding of most of the endometrial thickness. The endometrium comprises stromal, vascular and epithelial elements, all of which undergo cyclical changes

influenced by the ovarian hormone cycles of oestrogen and progesterone secretion, related to the process of ovulation. As ovulation occurs approximately mid-cycle and implantation a few days later, it is likely that the characteristics of the endometrium around this time must be at an appropriate "stage" for the successful reception of a fertilised ovum. It would seem reasonable to presume that the endometrium of fertile women share common characteristics which may be identified and which may be absent or deranged in the endometrium of some women in whom pregnancy appears unobtainable.

As has been suggested above, although not as yet amenable to diagnosis, the endometrium remains a prime site for abnormalities in relation to subfertility. Two studies up to now have suggested differences specifically in the endometrium of women with unexplained subfertility (Klentzeris et al. 1993; Lessey et al. 1995). It should be noted however, that unexplained subfertility can be primary or secondary, whilst in general study controls have been parous fertile women. The possibility that the endometrium of parous women may differ from that of nulliparous women has not previously been taken into account. Studies involving malarial parasitisation of the placenta in unigravid and multigravid women suggest that there is a permanent effect on utero-placental immunology exerted by previous pregnancy and thus the ability to withstand placental malaria resulting in a higher incidence of the disease in primiparous women (Rasheed et al. 1993). The factor of parity may affect other areas of endometrial function and therefore needs to be considered prior to further studies.

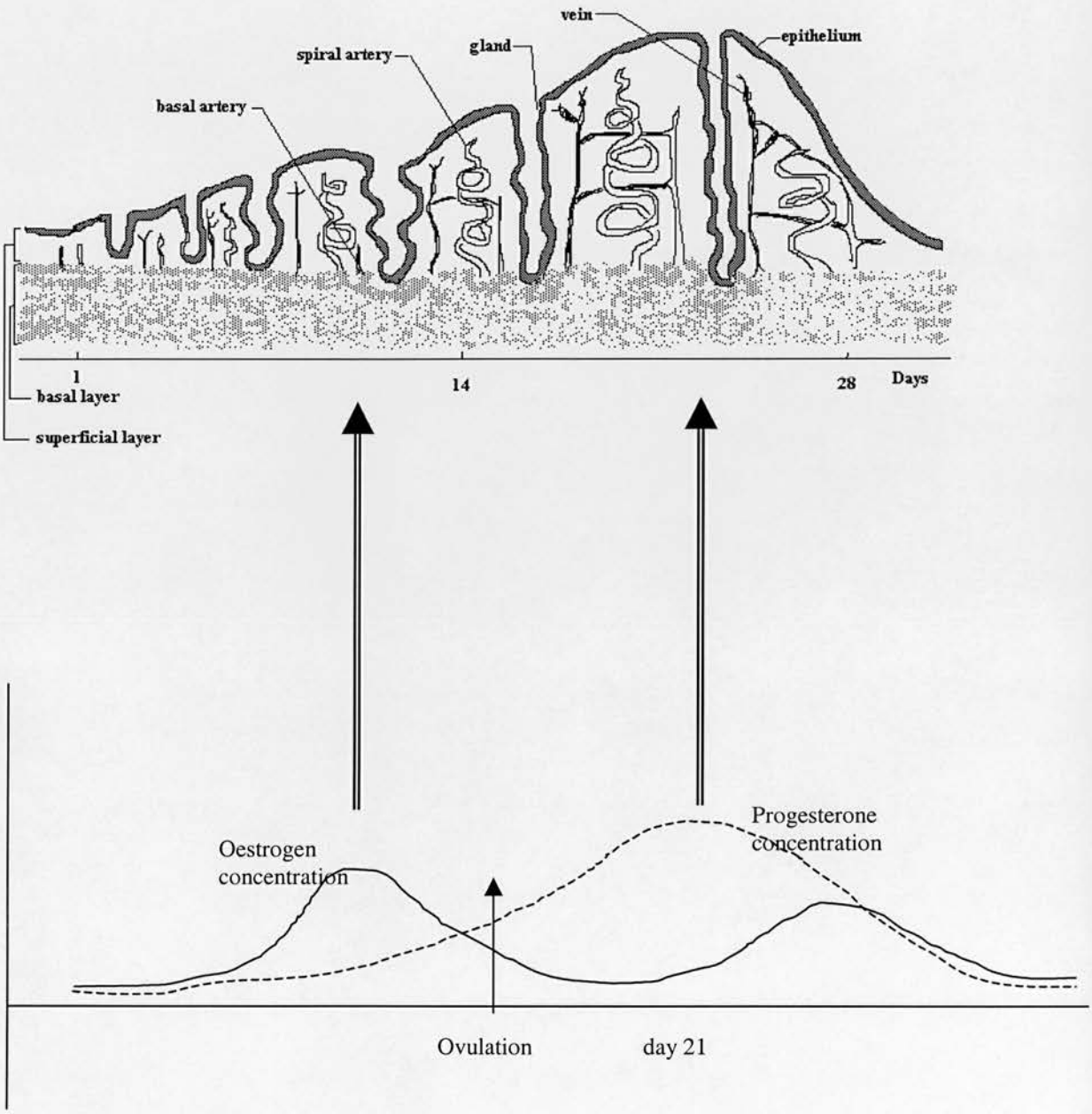
The Normal Endometrial Cycle (Fig. 1.3)

Immediately following menstruation only the basal layer of the endometrial stroma containing the bases of glands and shut down spiral vessels remains. The surface epithelium is absent. Although the basal layer of the endometrium undergoes some necrosis and leucocyte infiltration prior to menstruation, it is mostly protected from breakdown, presumably by supply from basal arterioles. Following menstruation and under the influence of rising oestrogen concentrations, the residual glands, the epithelium of which is initially low columnar, grows, producing pseudostratification. The epithelium grows out of the glands, reaching that of neighbouring ones and forming a continuous epithelial layer over the endometrial luminal surface. In addition the stroma enlarges and unbranched spiral vessels begin to extend into it.

Under the influence of oestrogen, in the so-called proliferative phase of endometrial development (Fig. 1.4a), the glands continue to grow having a straight, narrow appearance. Mitotic figures are seen in the glandular epithelium, which becomes tall. The stroma appears compact and cellular in this phase and again mitotic figures can be seen. The incorporation of water into the stroma leads to the increase in its thickness from 0.5mm to almost ten times that. Blood vessels grow freely through the stroma leading to a highly vascular appearance.

Oestrogen promotes the synthesis of progesterone receptors in the endometrium such that following ovulation, as ovarian progesterone production increases there is a change in the character of the endometrium during what is termed the secretory phase of the cycle (Fig. 1.4b). These changes are characterised by changes in the

Figure 1.3. *The endometrial cycle.*



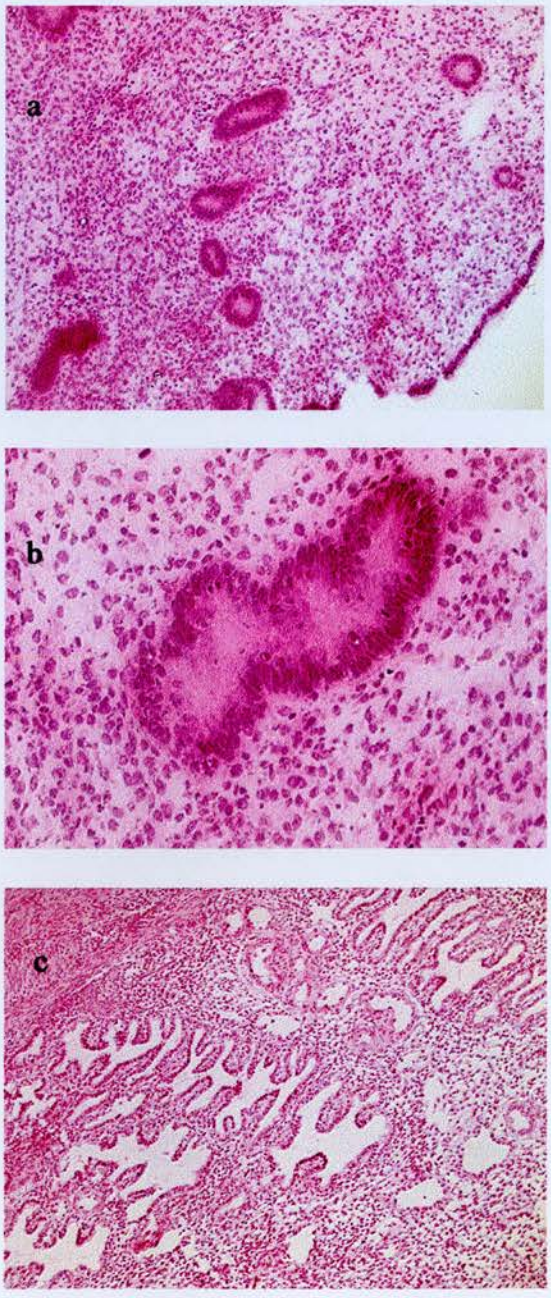


Figure 1.4. a) *Proliferative phase endometrium. H&E. (x20).* b) *proliferative phase endometrium. H&E (x40).* c) *Late secretory phase endometrium. H&E (x10)*

stromal, glandular and vascular elements of the tissue. The first observable change denoting ovulation and occurring 36-48 hours later is the development of subnuclear vacuolation in the glandular epithelial cells. These vacuoli disappear as the secretory phase progresses, nuclei returning to their basal position, and the glands become increasingly tortuous and dilated. Glycogen rich secretions appear in the lumen of the glands, which are maximal in amount by 10-11 days after ovulation. The stroma in the secretory phase of the cycle becomes increasingly oedematous. Oedema is the result of oestrogen dependent effects on the intracapillary hydrostatic pressure. The stromal cells themselves increase in size. At this stage the endometrial arterioles growing towards the endometrial cavity from the basal vessels appear to take an increasingly spiral course as they continue to grow without further increase in the endometrial thickness. At this stage there is a three layer appearance of the endometrium – the unchanged basal layer, the oedematous middle layer representing about 50% of the endometrial thickness (sometimes known as the stratum spongiosum) and the relatively dense superficial layer (stratum compactum). Together the luminal two layers form the functional endometrium. This three-layer appearance is reflected in the ultrasound appearance of endometrium in early secretory phase (Fig. 1.5a).

In a non-conception cycle, the corpus luteum regresses leading to falls in both progesterone and oestrogen concentrations. This results in a resolution of the stromal oedema and reduction of endometrial thickness giving the more compact appearance seen and the mid to late secretory phase appearance of dense endometrium on ultrasound scanning (Fig. 1.5b). This physical shrinkage results in the buckling of

Figure 1.5. *Transvaginal ultrasound scans of uterus demonstrating* a) *early secretory phase endometrium and* b) *late secretory phase endometrium.*

the spiral vessels which with accompanying vasoconstriction and relaxation results in ischaemia of the functional layer of the endometrium. Leucocyte infiltration occurs and overt necrosis follows. There is a natural break line between the relatively protected and compact basal layer and the "spongiose" portion of the functional layer of the endometrium and this subsequently sloughs off as part of the menstrual flow. Haemorrhage occurs from the exposed vessels but their vasoconstriction and formation of terminal clots, enhanced by oestrogen rising to commence the next cycle results in haemostasis and cessation of menstruation. Regeneration, originating from the basal layer, then proceeds.

If the oocyte derived from that ovarian cycle is fertilised, then implantation of the developing embryo occurs in the mid-secretory phase when the progesterone concentration and its influence are at their peaks. Successful conception and early embryo attachment results in continued progesterone secretion and endometrial decidualisation - notably persistence of stromal oedema and changes in stromal cell morphology. The stromal cells enlarge further giving a plump, polygonal appearance. Endometrial glands continue to increase in size with increased glandular secretion and there is continued capillary growth. These changes spread through the endometrium over the following days during implantation as secondary decidual changes.

Progesterone support of the endometrium continues well into the succeeding pregnancy as a result of the influence of human chorionic gonadotrophin (hCG) on the ovarian corpus luteum. Failure of fertilisation or implantation leads to atresia of

this structure, reduction in serum progesterone concentration, loss of hormonal support to the endometrium and its subsequent breakdown culminating in menstruation. Abnormalities of the implantation process would finally have a similar effect, with the possibility of an observable delay in menses. Early pregnancy failure results in miscarriage.

Endometrial Dating

The average human menstrual cycle is approximately 28 days and the endometrial changes occurring each day have been characterised: 1. In order to date an endometrial sample accurately; and 2. In order to identify any unexpected delay or acceleration in progression of the cycle, which could account for fertility problems. Noyes et al. (1950) in a much cited paper described the changes in endometrial histology based on dating from the next menstrual period - considered to be a more reliable landmark than the last menstrual period. Much debate has occurred over the subject of dating of the endometrium. It is generally agreed that this is most accurate and of most benefit when related to the time of ovulation rather than menses (either last or next). The rationale for this is that ovulation is a significant, fixed point in the hormone cycle upon which endometrial changes depend (Li et al. 1987; Shoupe et al. 1989). The problem with using ovulation as an anchor point for dating the endometrium is that there are no reliable overt signs for its identification compared with the onset of menstruation. Detecting the time of ovulation for the purposes of this thesis is discussed below (Clearplan- one step).

Previously much effort has been put into the assessment and treatment of "luteal phase" or "progesterone deficiency" (LPD) (Li and Cooke 1991). This diagnosis was based on the finding that endometrial biopsies, when dated histologically, were on occasion found to be "out-of-phase" with the actual time of the cycle at which the biopsy was taken. There is much contradictory evidence but as most of the histological dating in this work was based on Noyes' criteria (Noyes et al. 1950), and in most cases biopsies were timed from menses, much of the persuasiveness of the arguments is lost. Luteal phase progesterone secretory abnormalities have not been clearly demonstrated in relation to so-called LPD (Li et al. 1989b, 1990) and importantly the potential therapeutic effect of exogenous progesterone has not been confirmed in this situation. Finally a tendency to sample the endometrium as near to menses as possible developed, the rationale being that this would show the maximal cumulative effect of the "deficient" luteal phase.

Implantation

As ovulation occurs at approximately mid-cycle (day 14 of a 28 day cycle) and implantation of a developing embryo about seven days later it follows that during the secretory phase of the cycle the endometrium must be receptive to that embryo. In humans the embryo must attach to and invade the endometrium in order to establish the pregnancy.

The very earliest embryo to endometrial signalling was considered to be hCG secretion by the embryo on the first post-implantation day (Bergh and Navot 1992). Rudimentary evidence, however, for a pre-implantation embryonic influence on the

endometrium is available in mice (Das et al. 1994), a blastocyst dependent induction of heparin binding epidermal growth factor (HB-EGF) occurring in mouse endometrium prior to blastocyst adhesion.

Blastocyst adhesion involves major co-operation between embryo and endometrial epithelium and must provide the basis for many other interactions. Subsequent invasion of the endometrium by the blastocyst involves the break down of epithelial intercellular binding allowing trophoblast penetration to occur (Tabibzadeh and Babaknia 1995).

Window of implantation

The cyclical changes in endometrial histology have been discussed. It is known that human implantation occurs about seven days post ovulation at which time the endometrium must be considered to be receptive. There has been described, a window of opportunity for the embryo, during which time if it is appropriately developed it may adhere to and implant in the endometrial tissue (Bergh and Navot 1992). Outwith this time implantation is either not favoured or possibly directly inhibited leading to embryonic loss. The implantation window may involve many features of the endometrial cycle but adhesion is a major factor and has most precisely been described by Lessey et al. (1994) in terms of the changes in adhesion molecules expressed by endometrial stroma (Chapter 6).

Other features of the endometrium that relate to the implantation window are the appearance and disappearance of pinopodes and changes in the thickness of the

epithelial glycocalyx – increasing in thickness in humans. These aspects are reviewed by Giudice (1999).

Adhesion Molecules

Adhesion molecule is a general term used for a group of "families" of inter-cellular and some cell-substratum receptors (Abelda and Buck 1990; Fleming 1990; Garrod 1993). There are several families of adhesion molecules:

i) Cadherins - these are the major adhesion molecules of adhesive cell junctions. They are calcium dependent transmembrane glycoproteins and include neural (N-), epithelial (E-) and placental (P-) cadherin. N-cadherin is found in adult neuronal and muscle tissue and may have a developmental role in neuronal migration. E-cadherin plays an important role in early embryogenesis at the compaction stage, whilst P-cadherins are expressed in placental and epithelial cells and may be necessary for implantation. The glycoproteins making up the classical desmosomal plaque for cell-cell interactions are cadherins.

ii) Integrins – these are transmembrane glycoproteins made up of α and β subunits which determine their cytoskeleton linkage (β subunit) and ligand binding specificity (α subunit). α subunits, of which there are at least eight, may be associated by covalent linkage to different β subunits to produce integrins with specific binding qualities and functions (Fig.1.6). They perform cell-cell and cell-matrix binding functions. An integrin may bind several different ligands and a single cell type may express multiple integrins.

iii) Immunoglobulin super-family members – this group exhibits calcium independent binding that may be hetero- or homophilic. They are of particular importance in embryogenesis - neural cell adhesion molecule (N-CAM) for example; and immune cell antigen recognition and adhesion. Immune cell adhesion molecule-1 (ICAM-1, CD54) present on immune accessory cells has as its ligand the integrin receptor lymphocyte functional antigen-1 (LFA-1, $\alpha_L\beta_2$, CD11a) present on T cells, whilst vascular cell adhesion molecule-1 (V-CAM-1) is expressed by endothelial cells and binds very late antigen-4 (VLA-4, the integrin $\alpha_4\beta_1$).

iv) Selectins – this is a small family of adhesion molecules which unusually bind carbohydrates. They are intimately involved in the inflammatory response: for example, L-selectin which affects neutrophil adhesion to endothelium at sites of inflammation.

Thus adhesion molecules include a vast array of compounds which perform a variety of roles with regard to cell-cell and cell-extracellular matrix adhesion, but which, by virtue of these specific links also have the potential to provide important signalling functions.

The medical importance of adhesion molecules is becoming apparent as more is being discovered about them. For example, in pemphigus vulgaris, autoimmunity to desmosome cadherins develops, causing a breakdown of cell-cell junctions in keratinocytes of the skin and giving rise to blistering. Autoantibodies of a different specificity cause the disease of bullous pemphigoid as a result of failure of

epidermis-basement membrane adhesion. A variety of rare immunodeficiency diseases are known to be due to adhesion molecule abnormalities, for example, leucocyte adhesion deficiency (LAD) is due to lack of specific integrin subunits (Garrod 1993).

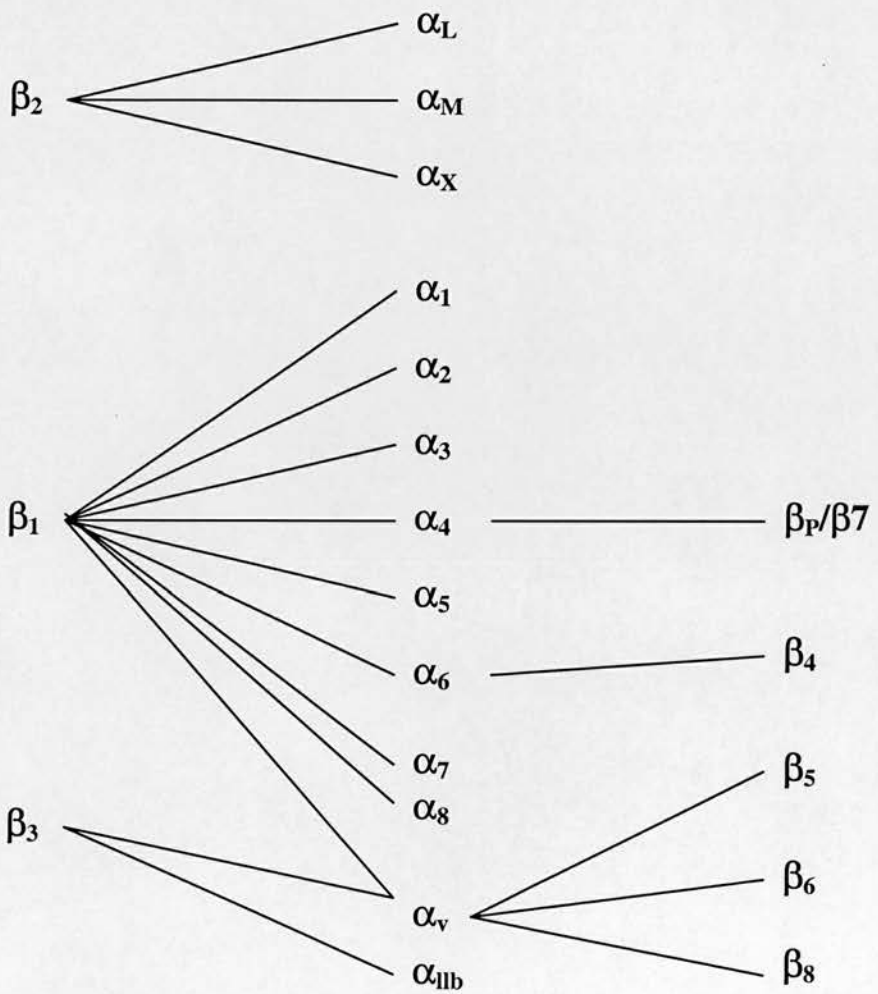


Figure 1.6. *Integrin subunit associations. (From: Abelda and Buck 1990; Hynes 1992)*

It seems reasonable to presume that amongst their vast array of functions and ubiquitous distribution, adhesion molecules would play a fundamental role in the function of the endometrium; its cyclical response, its immunology and importantly, its ability to attach, and maintain attachment to the developing embryo. Aberrant expression of such molecules in endometrial sites could lead to failure of these functions.

Endometrial Immunology

As foreign material, acceptance of an embryo must involve not only tolerance to that foreign phenotype but also control of its growth in order to prevent excessive invasion. This type of give and take suggests special modifications to the immune response of maternal endometrium in order to cope with the establishment of early pregnancy. Protection for the fetus against the maternal immune response is conferred by the absence of classical major histocompatability (MHC) antigens on trophoblast surfaces thus avoiding MHC dependent T cell activity (Johnson and Christmas 1996). The invasive extravillous cytotrophoblast, expresses a class of antigen (HLA-G) not seen in adult tissues (Kovats et al. 1990, Ellis et al. 1990) which although remaining inert to T cell detection, conveys enough identity to protect against the "absent self" responses of natural killer (NK) cells (Ljunggrens and Kärre 1990). Trophoblast also expresses cluster of differentiation (CD) 46, CD55 and CD59, complement regulatory proteins which protect against antibody-mediated attack (Johnson and Christmas 1996). Fetal immune cells, once developed, are separated from maternal tissues by the trophoblast barrier thus avoiding any fetal to maternal immune reaction.

The immune changes occurring in the endometrium throughout the menstrual cycle have been described in detail (Starkey et al. 1991, Bulmer et al. 1991b, Klentzeris et al. 1992). The stromal leucocyte populations in human endometrium vary as the cycle progresses and it is because of these marked changes in leucocyte populations and the need to invoke immunological factors in the process of implantation that the importance of endometrial leucocytes and fertility has become an issue.

Endometrial granulated lymphocytes

Endometrial granulated lymphocytes (eGLs) or large granular lymphocytes (LGLs), more recently termed "uterine NK cells" are a specific group of cells found in the endometrium and decidua. Originally considered to be derived from stromal cells and known as Körnchenzellen or "K" cells (Hamperl 1955, Hamperl and Hellweg 1958, Dallenbach-Hellweg 1981), eGLs were later identified by Bulmer et al. in 1983 and using immunohistochemistry, shown to express the leucocyte common antigen (CD45) confirming their bone-marrow origin.

These cells possess small oval or indented hyperchromatic nuclei and contain phloxinophilic cytoplasmic granules (Pace et al. 1989). They are found throughout the endometrial stroma and in decidual tissue either scattered singly or in small aggregates associated with endometrial glands or spiral arteries (Klentzeris et al. 1992, Bulmer et al. 1991a).

In the non-pregnant endometrium there is a cycle stage dependent increase in the numbers of eGLs, occurring between LH+7 and LH+13 (denoting 7 and 13 days

respectively, from the luteinising hormone surge), such that although at LH+4 eGLs make up 45% of endometrial leucocytes, the proportion of leucocytes which are eGLs rises to 55% at LH+13 (Bulmer et al. 1991b; Klentzeris et al. 1992) (Fig. 1.7).

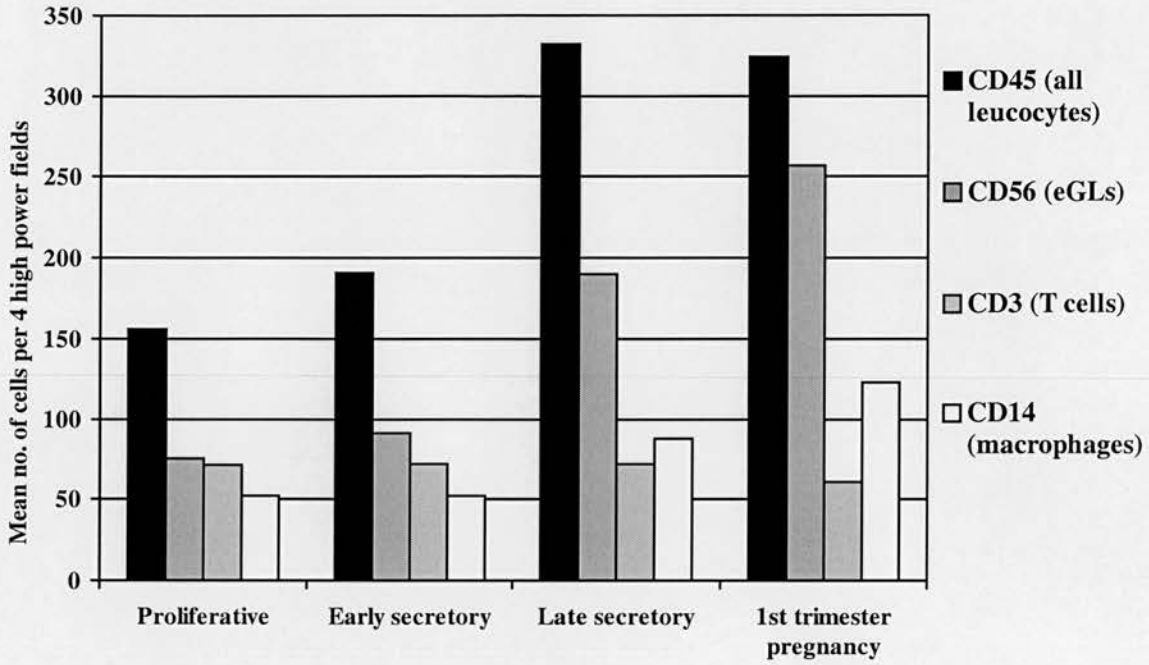


Fig. 1.7. *Endometrial leucocyte distribution throughout the menstrual cycle and early pregnancy (Bulmer et al. 1991b).*

Large numbers are "shed" at menstruation although it has been suggested as a result of their changing appearance at this stage that they may be undergoing apoptosis premenstrually (King et al. 1989).

In first trimester decidua around 75% of endometrial leucocytes are eGLs (Bulmer et al. 1991b) but there is a general decline in numbers as pregnancy progresses. Bulmer and Sunderland (1984) and Bulmer et al. (1987) characterised these cells to be of

early T cell lineage expressing CD2 (E-rosette receptor). They fail however, to express other major mature T cell markers; CD3, CD4, CD5 and CD8. They react with OKT 10, a B and T cell marker which is not present on mature lymphocytes and CD7 the T cell differentiation antigen. Further characterisation of their phenotype by Ritson and Bulmer (1987), Pace et al. (1989) and King et al. (1991) [Appendix 1] has led to the finding that they are similar to a small subset of peripheral blood NK cells. It is of interest that the murine equivalent cells – granulated metrial gland cells – have in gene-ablated mice been assigned NK cell lineage (Croy et al. 1997), and that their absence results in implantation abnormalities (Guimond et al. 1997). There is no known human deleted equivalent.

The origins of the increased population of eGLs are considered to be mainly by local proliferation. The evidence for this comes from the fact that emigration from peripheral blood, the logical alternative, would be presumed to give cells with a similar phenotype. The eGL phenotype is virtually exclusive to endometrium and comparable cells are rare in peripheral blood. In addition, examination of endometrial tissue has shown that although there are few mitotic figures seen in the population, the co-expression of Ki67 (a product of cell replication found in any cells not in G_0 phase) increases as the cycle progresses (Pace et al. 1989, Tabibzadeh 1990a, King et al. 1991). This is in keeping with the observed increase in population numbers. Klentzeris et al. (1992) suggest that this is supported by their findings that CD2 expression in eGLs decreases as the cycle progresses, perhaps as a lag in its manufacture in a rapidly expanding population, resulting in a larger proportion of less well differentiated eGLs in the later stages of the cycle. The control of this

proliferative activity has yet to be elucidated. Loke and King (1995) suggest that both oestrogen and progesterone are essential for their presence in the endometrium of ovariectomised women. It is likely that there are local secondary factors which may be under overall hormonal control, although Klentzeris et al. (1992) argue that the biggest rate of proliferation of eGLs occurs when secretory phase steroid hormone concentrations are waning premenstrually. Critchley et al. (1996) however, found no significant change in the numbers of CD56 positive cells in decidua following the administration of the antigestogen RU486 *in vivo*.

It is possible that circulating eGL-like peripheral blood NK-cells represent a transfer of bone marrow-derived cells to the endometrium as a topping-up process or as a source of precursor for subsequent population expansion (King et al. 1991). Evidence for the recruitment of leucocytes from the endometrial vascular system comes from the findings that there are larger numbers of eGL-like peripheral blood NK cells in females than males (King et al. 1991) and that specific chemoattractant agents (interleukin-8 [IL-8], monocyte chemoattractant protein-1 [MCP-1] and cyclo-oxygenase-2 [COX-2]) have been located at perivascular sites at crucial times of the cycle (Jones et al. 1997b).

The functions of eGLs are poorly understood but have been mostly studied in cells derived from early pregnancy decidua rather than non-pregnant endometrium. They are known to exhibit a degree of activation; 40% express CD69 and 20% are HML-1 positive (King et al. 1991). These are known leucocyte activation markers. In addition eGLs can be made, *in vitro*, to exhibit NK-type functions and are

interleukin-2 (IL-2) responsive, probably via the IL-2Rβ sub-unit which they express (Starkey et al. 1991). They are cytotoxic to the NK cell target, K562 cell line, a function enhanced by IL-2 stimulation. They can be made, by prior incubation with IL-2 to become cytotoxic to BeWo and JEG-3 choriocarcinoma cell lines, fetal fibroblasts and normal human trophoblast (King and Loke 1990a, Ferry et al. 1991). This is a phenotype dependent observation; Christmas et al. (1990), found that CD16 positive cells had high cytotoxicity whilst that of CD16 negative clones was low. Cytotoxic clones were almost all CD2 positive. Non-cytotoxic clones were CD2 negative.

NK cell activity is HLA independent. NK cells detect cells that do not possess MHC molecules that is cells that are "non-self" (the "missing self" hypothesis) (Ljunggren and Kärre 1990). They are relatively indiscriminate therefore with respect to foreign cells. In their similarity to NK cells it can be seen that eGLs may or may not play a role in the control of trophoblast invasion of the endometrium. Deniz et al. (1994) also found that CD16 negative eGLs had lower cytotoxic activity than their peripheral blood equivalents against Molt 4 and BSM cell lines, suggesting a role for the CD16 antigen but in addition they found that the presence of HLA-G appeared to significantly protect cells from eGL cytotoxicity, protection which is not afforded against peripheral blood cells. Furthermore, Bulmer et al. (1984) reported that eGLs are abundant in decidua where trophoblast invasion is least prominent and they are not seen in large numbers at the site of tubal ectopic implantations unless there is decidualisation of tubal mucosa, whilst they are present in the uterus in this condition. Thus the direct effect of eGLs on trophoblast control seems less likely.

Deniz et al. (1994) also reported that eGLs could exert an effect on an *in vitro* mixed lymphocyte response (reported to be inhibitory using freshly prepared clones) whilst Christmas et al. (1990) have shown the production of interferon γ (IFNγ), tumour necrosis factor α (TNFα) and transforming growth factor β (TGFβ) in varying amounts by eGL clones. This supports the idea that these cells may act in a regulatory fashion within the endometrium rather than through a direct action on trophoblast. Clarke et al. (1994) have confirmed eGL production of TGFβ *in vitro*, and Saito et al. (1993) detected mRNAs coding for granulocyte colony stimulating factor (G-CSF), granulocyte and macrophage colony stimulating factor (GM-CSF), macrophage colony stimulating factor (M-CSF), TNFα, IFNγ and leukaemia inhibiting factor (LIF) in eGLs isolated by cell-sorting from first trimester decidual tissue. Cultures of these cells produced the respective proteins, detected by enzyme-linked immunosorbent assay (ELISA) and bioassays, thus lending further weight to the idea of a regulatory role.

Thus eGLs are endometrial stromal cells of bone marrow origin with NK cell-like features, and which proliferate in the secretory phase of the menstrual cycle and persist into early pregnancy at decidual sites both at and distant from the site of implantation. The control of their numbers and their functions in this situation are not fully elucidated. Whilst direct cytotoxic effects on trophoblast may appear less likely, a regulatory function could be most important.

Endometrial T cells

Endometrial T cell numbers are believed to be relatively constant throughout the menstrual cycle (Starkey et al. 1991, Klentzeris et al. 1992) (Fig. 1.7). The majority of endometrial T cells are cytotoxic / suppressor type (CD8 positive) with a CD8/CD4 ratio of 2.8, again believed to remain fairly constant throughout the cycle (Klentzeris et al. 1992). Most T cells in the endometrium express T cell receptor αβ (TCRαβ) subtype with only a small minority expressing the γδ antigen (Vassiliadou and Bulmer 1996). αβ positive T cells are scattered throughout endometrial stroma and form small aggregates towards the basal layer of the endometrium (Klentzeris et al. 1992). Their specific functions are unclear; however, as unlike eGLs their phenotypes are common to T cells at other sites and in the blood and not peculiar to the endometrium then normal T cell functions might be expected. Whether or not T cells contribute directly to the control of implantation is debatable. T cells by virtue of a wide range of soluble products, which act locally, are capable of co-ordinating and modulating the local immune response. Typically they recognise antigens presented in relation to class II MHC molecules on a cell's surface and respond appropriately according to their sub-type.

Trophoblast in contact with decidual tissue does not, as discussed earlier, express classical class I or class II MHC antigens and consequently cannot be directly responsible for eliciting an aggressive αβ positive T cell response. Thus a direct effect of αβ positive T cells on implantation is unlikely.

Wegmann et al. (1993) discussed the likely effects of the balance of T cell functions in the uterus of the mouse. They suggested that the production of the correct balance of T cell derived cytokines is crucial to embryo survival. So-called T_H1 cytokines (IL-2, IFNγ and TNF) produced by some helper T cells are considered to be generally harmful to pregnancy maintenance. They hypothesised that IL-4, 5, 6 and 10, coined T_H2 cytokines, protect a conceptus which produces them by down-regulating the production of T_H1 cytokines. In mice these cytokines have been detected in supernatants of decidual cell cultures, implying production at the feto-maternal interface (Lin et al. 1993). T_H2 cytokines are involved in B cell development, favoring the humoral immune response: T_H1 cytokines favour the cytotoxic immune response. This may be reflected in altered immunology generally in pregnancy resulting in exacerbation of humoral dependent auto-immune conditions e.g. systemic lupus erythematosis (SLE) and improvement of conditions dependent primarily on the cell mediated response e.g. rheumatoid arthritis. It is postulated that any local stimulus resulting in an imbalance of these two groups of cytokines, for example infection favoring a cell-mediated response might affect implantation and early pregnancy survival.

Further to these discussions, it is clear that local cytokines may affect NK cell activity, for example via IL-2 production. There is some evidence that peripheral blood NK cells are effectors in the induction of cytotoxic T cells from precursor to effector cells – a function of the CD56 antigen which is blocked by anti CD56 monoclonal antibodies (Kos et al. 1995). It is possible that a similar influence by CD56 positive eGLs may prevail in the endometrium also.

The function of γδ positive T cells in the endometrium is unknown. They are capable of non-MHC restricted killing and in mice, whilst present in greater numbers than in human endometrium, are capable of trophoblast recognition (Heybourne et al. 1994). A role in trophoblast / endometrial interaction has not been determined in humans but in this situation abnormal function could potentially result in altered fertility.

Endometrial macrophages

Macrophages comprise 10-30% of endometrial leucocytes (King et al. 1991, Klentzeris et al. 1992) (Fig. 1.7). Various opinions have been given with regard to their numbers throughout the menstrual cycle. Starkey et al. (1991) found them to remain fairly stable in number until menstruation when their numbers increased, whilst Klentzeris et al. (1992) found their numbers to increase in the late secretory phase. There is no evidence to suggest any endometrial sub-specialisation and although highly activated in pregnancy, their functions would be expected to be similar to those of macrophages at other sites providing a non-specific defence against infection (Johnson and Christmas 1996). The perimenstrual increase in numbers may be related to the structural endometrial remodelling required during and following menstruation. Many will be lost during menses reducing the numbers for the start of the next cycle.

Endometrial B cells

There are very small numbers of scattered B lymphocytes in the endometrium. There appears to be no real fluctuation in their numbers. Any specific endometrial

functions have not been evaluated and any increase in their numbers is likely to be related to infection.

Embryo Influences on Endometrial Immunology

As has been suggested above even pre-implantation embryos may have specific effects on the endometrium. In addition once adhesion has occurred there is set in train one of the most complex systems of co-operation between what are essentially two separate organisms. It is well known that the developing embryo and fetus initially promotes its own survival by the secretion of hCG which results in maintenance of the ovarian corpus luteum and its secretory function – mainly progesterone production. What is much less well understood are the local interactions between embryo and endometrium. The physical attachment and invasion of the embryo have been considered but adhesion molecules have messenger functions as well as structural and this attachment process may give rise to a cascade of downstream events. In addition the embryo is a source of cytokines – local messenger molecules which may well have a modulatory function on the endometrium. Previous attempts have been made to identify proteins secreted by pre-implantation embryos. The effects of these proteins *in vivo* on endometrial function, in particular immunological function, are unknown. This aspect of fertility is discussed more fully below (Chapter 7 – The Effects of Embryo Derived Factors on Endometrial Leucocytes).

Endometrial biopsy

When required endometrial biopsy, although an invasive procedure, is relatively simple and safe to perform as an outpatient (Li and Cooke 1990). Outpatient endometrial sampling is most usually used in younger women with dysfunctional uterine bleeding where hysteroscopy is not mandatory, or in older women with postmenopausal bleeding in conjunction with vaginal ultrasound scanning. There is as yet insufficient evidence to support the routine use of endometrial sampling as part of the work-up for fertility assessment (RCOG 1998).

A variety of disposable samplers are available, but the sampler favoured for the following studies was the Pipelle endometrial sampler (Euro Surgical Ltd., Guildford, UK) (Fig. 1.8), which can be inserted through the cervical canal without analgesia with a minimum of dysmenorrhoeic type discomfort for most women. The endometrial biopsy is obtained by withdrawing the central core of the sampler producing a degree of suction adequate to aspirate endometrium through the side port near the tip whilst moving it around the uterine cavity [Appendix 2]. About 1ml of sample can be obtained, although smaller samples would be produced if an attempt was made to direct the biopsy to a particular site in the uterus. Good volume samples can be obtained and processed immediately (Chapter 2 – Materials and Methods).

Vaginal spotting may succeed sampling and subsequent menstruation is usually normal. Complications, which potentially include uterine perforation and infection, are rare (Li and Cooke 1990).

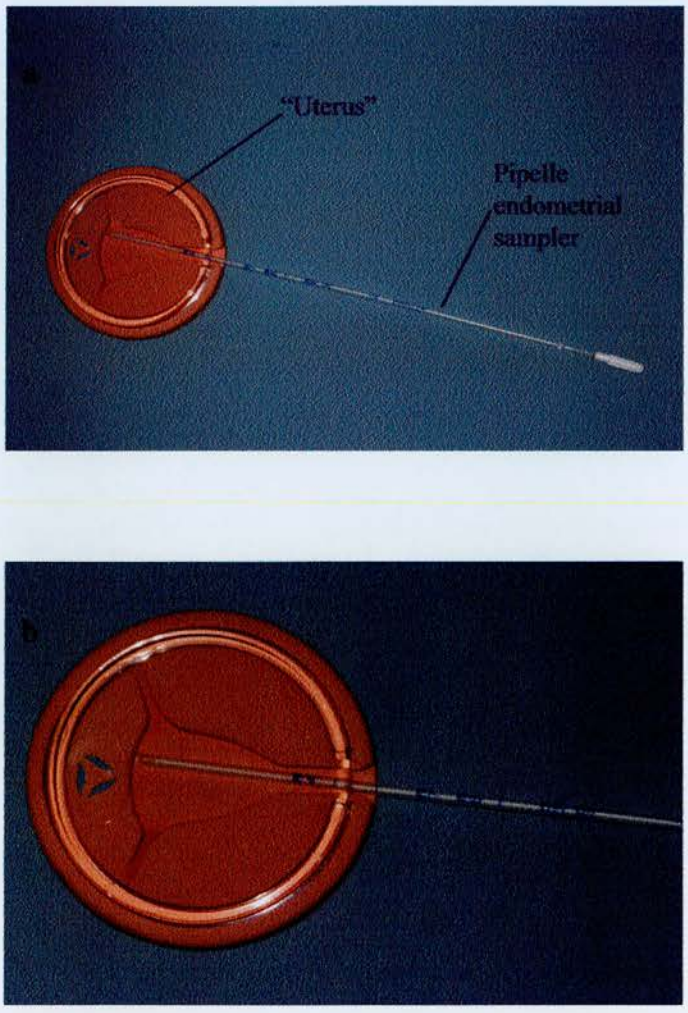


Figure 1.8. a) *Pipelle endometrial sampler,* b) *indicating insertion into model uterus.*

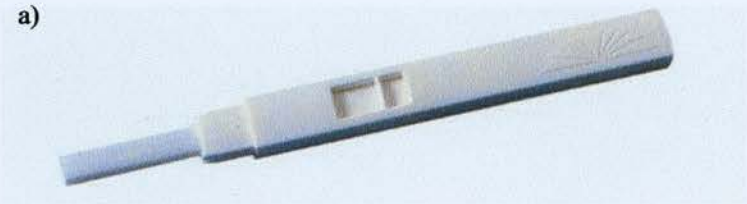
Timing of endometrial samples

In order to correctly sample the uterus and make sense of the histological findings when the biopsy is examined, its timing needs to be related to ovulation. Various markers for this have been used previously – basal body temperature (BBT) changes, ultrasound scan follicular tracking, last menstrual period or luteinising hormone (LH) surge, either urinary or serum. For the purposes of the present studies urinary LH surge identified by Clearplan (Unipath, Bedford, UK) kits was used (see below). Ultrasound and identification of the serum LH surge are the best markers of ovulation but would have required an unacceptable number of hospital visits for subjects.

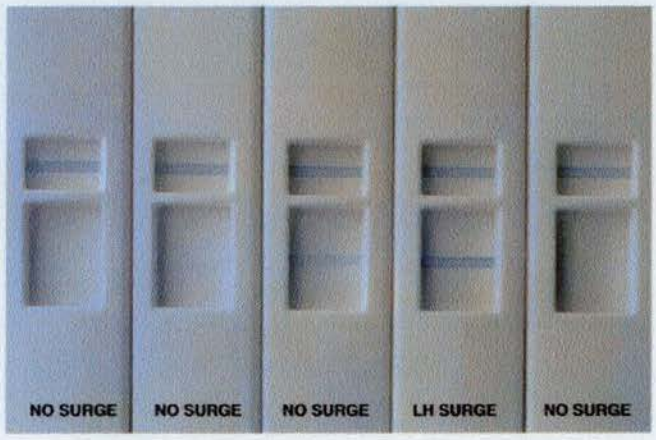
Clearplan – One Step

This urine test kit is marketed to the public as an ovulation predictor kit, designed to enhance a couple's chance of conceiving by making them aware of the "fertile period" (Fig. 1.9a). It is designed to detect the onset of the LH surge occurring 32 to 38 hours before ovulation and which is approximately coincident in serum and urine (Corsan et al. 1990). LH is a di-peptide glycoprotein sharing an α sub-unit structure with those of follicle stimulating hormone (FSH), human chorionic gonadotrophin (hCG) and thyroid stimulating hormone (TSH). The β sub-unit determines the biological activity and is structurally and functionally distinct from the other glycoprotein hormones, although hCG and LH β subunits share 85% sequence homology and potentially therefore cross-react.

a)



b)



LH surge = line in the large window is as dark as or darker than the line in small window.
No LH surge = no line in large window, or line paler than line in small window.

Figure 1.9. *Clearplan - One Step.* a) *test device,* b) *interpretation of result.*

Clearplan One-Step is basically an immunoassay using chromatography to separate bound and free coloured label. Urine moving along the device first comes into contact with mouse monoclonal antibody to the β subunit of LH, which binds to the hormone if present and is mobilised into the urine. This antibody is labelled with blue latex. The second antibody is fixed below the test window and binds the α subunit of LH, thus immobilising the hormone and its blue latex labelled primary antibody, producing a blue line. The test incorporates a control window to ensure proper use of the kit. This contains fixed anti-mouse antibody which will pick up excess and unbound anti-LH antibody drawn along the device in the urine and which, bearing the blue latex will again produce a blue line. This line will only develop therefore if urine has passed along the whole device ensuring that there is adequate coverage of the test window. It can be seen that this window will contain a blue line even in the absence of LH in the urine sample and thus acts as a control (Fig. 1.9b). A kit where a blue line does not develop in the control window cannot be considered valid as a false negative test may result.

Schmultzer et al. (1994) described the use of Clearplan kits in stimulated and spontaneous ovarian cycles and suggested a specificity of 100% and sensitivity of 97.5% (100% and 100% respectively in spontaneous cycles) for its detection of the LH surge. It has however, been shown to be less efficient than laboratory urine LH testing by Anderson et al. (1996) in a program for donor insemination treatment. It is likely that personal motivation is important in the appropriate use of the kits and this may differ in different situations.

Aim of This Thesis

The aim of this thesis is to establish more information about the significant problem of unexplained subfertility. It can be seen from the above discussion that the endometrium is a highly complex and dynamic structure. It is unlikely therefore, that it serves purely as a passive recipient of an embryo for implantation. There is growing evidence that specific endometrial features are required to be present around the time of implantation and there is therefore the possibility that abnormalities in the cyclical events of the endometrium may affect fertility.

To this end the hypotheses which have been tested are;

- endometrial immunological changes are directly controlled by ovarian sex steroids;
- endometrial cell proliferation and demise are altered in unexplained subfertility;
- endometrial leucocyte populations are altered in unexplained subfertility;
- endometrial adhesion molecule distribution is altered in unexplained subfertility;
- previous parity affects endometrial immunology and adhesion molecule distribution; and
- embryo derived factors influence the function of endometrial leucocytes.

CHAPTER 2

MATERIALS AND METHODS

This chapter describes the processing of specimens for the subsequent studies and includes a discussion of subject recruitment. The methods used to examine specimens are described as are the techniques used for the statistical analysis of the results. The methodologies for specific studies are described in the appropriate chapters' experimental design.

1. SAMPLE ACQUISITION

Timed Endometrial Pipelle Biopsies (frozen) (Chapters 3,5 and 6)

RECRUITMENT

In this chapter Pipelle samples were obtained from volunteers in each of four groups:

1. Nulliparous infertile (unexplained) and 2. Parous infertile (unexplained).

These women were recruited by written invitation sent to all those with unexplained subfertility on the National Health Service (NHS) waiting list for assisted reproductive treatment at the Centre for Reproductive Medicine, Royal Victoria Infirmary, Newcastle-upon-Tyne. One hundred and eighty one women with unexplained infertility of at least two years standing were approached by letter: fifty-five replied, thirty-two positively. Six other subjects were recruited from clinics and by word of mouth. Recruiting from clinic was not very fruitful because many women, particularly nulliparous women, were seen with less than two years' subfertility and were therefore excluded. Of those who had had at least two years' problems, parous women were excluded from NHS treatment and were therefore either undertaking private treatment at the first opportunity or would not be undergoing treatment and having no wish to return to the unit could not be recruited.

All women recruited to groups 1 and 2 had had basic fertility investigations performed including confirmation of ovulation and confirmation of tubal patency and exclusion of endometriosis. Their partners had submitted normal sperm samples.

3. Nulliparous fertile (presumed fertile)

These women were recruited mainly by word of mouth. A few volunteered following application to pre-clinical medical students. Three were recruited from the donor insemination programme having had ovulation and tubal patency confirmed but as there was no waiting list for donor insemination, recruiting from this source was limited as participation would entail at least a two to three month delay in commencing the treatment program. These women as a group have clearly not proceeded as far along their reproductive path as those of the other groups, a factor reflected in their average ages (Table 2.2).

4. Parous fertile (proven fertility).

These women came from family planning clinics and sterilisation waiting lists, again by written invitation and also by word of mouth from subfertile subjects, associates etc. Women from the "fertile" groups were excluded if any form of hormonal treatment including the oral contraceptive pill or an intra-uterine contraceptive device (IUCD) were used as forms of contraception.

Nulliparous women had had no pregnancies (including miscarriages and terminations – nulligravid). Parous women had had at least one pregnancy delivering after 24 completed weeks.

In total sixty-four women were consented into the subject groups (Tables 2.1 and 2.2).

Twenty-three women defaulted from the study, fifteen prior to any sampling and eight following a single sample. Only one woman formally withdrew prior to the study. One subject was withdrawn because of poor tolerance of the first sample, one withdrew because of insurmountable problems interpreting Clearplan kits, and three withdrew to commence fertility treatment. Five nulliparous women were withdrawn because of difficulties passing the Pipelle sampler. Although their notes were annotated as such, of the four subfertile women in whom sampling was impossible two have subsequently undergone IVF and embryo transfer (ET) without difficulty. ET catheters are narrower gauge, more flexible and the need to insert them despite discomfort is clearly greater, hence the discrepancy.

Table 2.1. *Recruits to fertility/unexplained subfertility studies.*

	Total Recruited	**Number of samples submitted**		
		2	**1**	**0**
Nulliparous fertile (NF)	12	5	2	5
Nulliparous infertile (NI)	27	8	6	13
Parous fertile (PF)	11	8	2	1
Parous infertile (PI)	14	5	5	4

Table 2.2. *Details of recruits to fertility/unexplained subfertility studies.*

	Mean age–years (range)	**Mean cycle length–days (range)**	**Mean parity**	**No. of LH+7 samples**	**No. of LH+13 samples**
Nulliparous fertile (NF)	24.6 (19 – 30)	29.1 (24 – 35)	0+0	7	5
Nulliparous infertile (NI)	32.3 (28 – 37)	28.9 (26 –35)	0+0	12	10
Parous fertile (PF)	33.8 (27 – 40)	29.1 (26 – 35)	2+0	9	9
Parous infertile (PI)	34.9 (29 –39)	28.2 (25 – 32)	1+0	10	5

ENDOMETRIAL SAMPLING

Pipelle samples (Appendix 2) from these subjects were obtained on days LH+7 and LH+13 in separate cycles where LH+0 is the day of onset of the LH surge detected by Clearplan urinary test kits (Introduction). Four women failed to provide an LH+13 sample because of "early" menses commencing on day LH+13 on one or more occasion. All subjects providing endometrial Pipelle samples received an information leaflet and gave their informed consent [Appendix 3]. Unless previously sterilised, all were advised to use condoms during the test cycles in order to avoid sampling a conception cycle. There were no complications of sampling other than vaginal spotting in any of the subjects.

TISSUE PROCESSING

Once obtained, endometrial samples were each extruded from the Pipelle sampler into a foil formed cup then immediately snap frozen in liquid nitrogen cooled isopentane (methyl-2-butane, BDH Chemical Company Ltd., Poole, UK) to form a

frozen tissue block. Samples were stored in cryotubes (Bibby Sterilin, Stone, UK) at - 70°C.

6μm cryostat (Reichert Jung) sections of endometrium from each sample were cut onto poly-L-lysine (Sigma Chemical Co., Poole, UK) coated slides and air-dried overnight at room temperature. They were fixed in acetone (BDH) for ten minutes, again at room temperature and stored, foil wrapped, at – 20°C until used.

Haematoxylin and eosin (H&E) staining was performed on sections of all tissues to ensure that an adequate amount of endometrium, with well-preserved morphology was present (Fig. 2.1).

Tissues Derived From Pathology Specimens (Chapter 4)

FROZEN BLOCKS

In Chapter 4 reference is made to samples of endometrium derived from hysterectomy specimens and from first trimester terminations of pregnancy. Blocks, 5-10mm cubes of endometrium with attached myometrium to allow good orientation, were snap-frozen in liquid nitrogen cooled isopentane. Decidual tissue, identified macroscopically by its opaque grey-white, solid appearance, was obtained from early gestation suction terminations of pregnancy with fully informed consent. Decidual tissue was irrigated in normal saline to remove excess blood and snap-frozen in a similar way in 5-10mm cubes. All blocks were stored at -70°C. Storage and preparation of slides was similar to that already described.

H&E staining was performed to check orientation at cutting to ensure that an adequate amount of tissue with normal well preserved morphology was included in sections (Fig. 2.1).

PARAFFIN-EMBEDDED BLOCKS

Paraffin sections were prepared from archival blocks of endometrium and decidua. They were cut onto 3-aminopropyltriethoxysilane (APES) (Sigma) coated slides at a thickness of 3μm.

Embryo supernatants (Chapter 7)

The acquisition of embryo supernatants and subject selection for this is discussed in full in Chapter 7.

2. IMMUNOHISTOCHEMISTRY

Preparation

Frozen sections

Frozen sections were unwrapped, allowed to warm to room temperature and rehydrated prior to immunohistochemical processing.

Paraffin-embedded tissue

After the sections were deparaffinised in xylene (BDH) and rehydrated in graded alcohols, antigen unmasking was achieved by microwaving (800W, Panasonic) for 2 x 5 minutes either in 0.01M sodium citrate buffer (pH 6.0) (BDH) (Norton 1993, Cattoretti et al. 1993) or, for ER-6F11 (see Table 4.1), in 1mM ethylenediaminetetra-

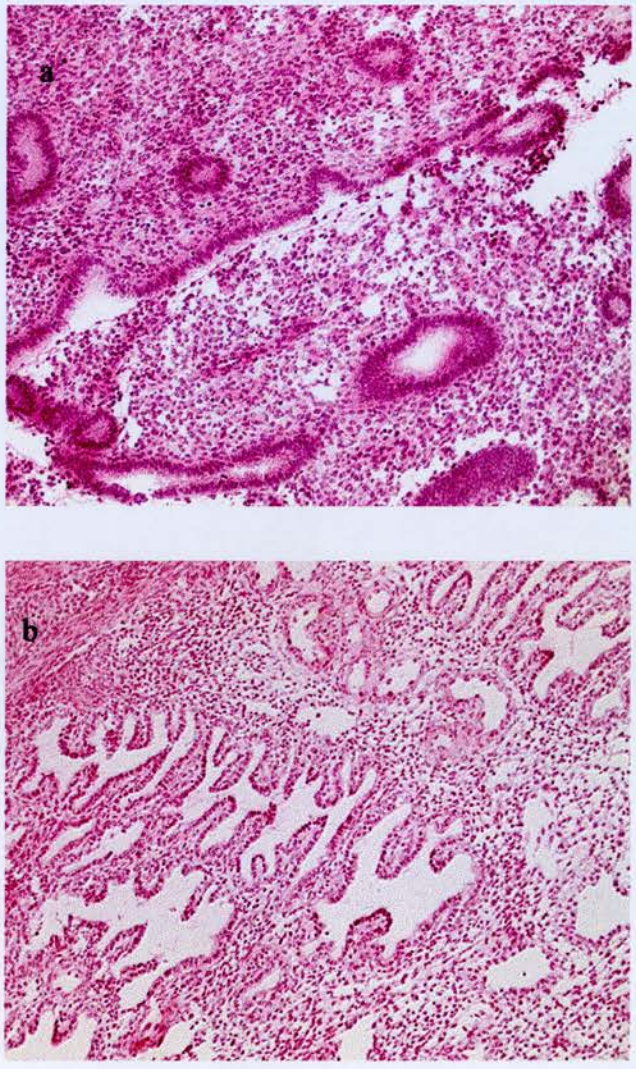


Figure 2.1. a) *Pipelle sample of endometrium H&E. (x20).* b) *Frozen section endometrial block. H&E. (x10).*

acetic acid (EDTA) buffer (pH 8.0) (BDH). After cooling, immunohistochemical staining was performed as outlined below.

Single Immunohistochemical Labelling (Chapters 3, 4, 5 and 6)

Single immunohistochemical labelling of frozen sections was performed using the Vectastain Elite ABC kit (Vector Laboratories, Peterborough, UK) and secondary and tertiary reagents were prepared according to the kit instructions given. This kit is based on the avidin-biotin-peroxidase complex (ABC) immunohistochemical technique described by Hsu et al. (1981). The procedure was carried out at room temperature except where otherwise stated. After initial rehydration for 10 minutes in 0.05M Tris buffered 0.15M saline pH 7.6 (TBS) (Sigma), sections were overlain for 30 minutes with non-immune serum (provided as a reagent in the kit) from the same species as that in which the secondary antibody was raised. Its purpose is to block non-specific binding by excess antibody. Normal serum was then drained off and the slides incubated with primary monoclonal antibodies (mAb) at the appropriate dilution in TBS for 60 minutes. After washing in TBS, biotinylated secondary antibody was applied for 30 minutes followed by further washing and incubation with ABC reagent (an avidin and biotinylated peroxidase preparation) for 30 minutes. The detection of labelling with primary monoclonal antibody in this assay is dependent on the ability of avidin, an egg white protein to bind multiple biotin molecules (there are four binding sites per avidin molecule). As multiple biotin molecules can be bound to a single peroxidase molecule a complex structure of avidin, biotin and peroxidase can result based on a single antibody molecule. This leads to a major amplification of apparent labelling that can then be visualised by

conversion of a peroxidase substrate to a coloured product. Thus a third wash in TBS was followed by incubation with the peroxidase substrate, 3-3' di-aminobenzidine (0.1%, DAB, Sigma) prepared in TBS with 0.01% hydrogen peroxide (H_2O_2, BDH) giving a brown reaction product (Chapters 3, 4, 5 and 6). In Chapter 4 amino-ethyl carbazole (AEC) (Vector), supplied as a kit reagent was also used as a peroxidase reagent, producing a red reaction product. The reaction was monitored microscopically to avoid excessive colour development that would obscure the tissue morphology, and stopped after approximately 5-10 minutes for DAB, and approximately 15 minutes for AEC, by washing in excess water. The sections were lightly counterstained with Mayer's haematoxylin producing purple-blue nuclear staining for morphology.

Controls

Negative controls, excluding primary antibody were included in all immunohistochemistry runs to assess background staining and non-specific labelling. Positive controls included frozen sections of human tonsil for leucocyte antigens and adhesion molecules whilst breast tumour tissue provided the positive control for steroid hormone receptors.

Double Immunohistochemical Labelling (Chapter 3)

Double immunohistochemical labelling was performed in order to detect co-expression of steroid receptors by endometrial leucocyte populations. Sections were initially labelled for leucocyte antigens with the ABC method described above using AEC. After development of the AEC red reaction product, sections were washed in

TBS for 10 minutes, overlain with blocking serum for a further 30 minutes and incubated with the second primary monoclonal antibody directed against oestrogen receptor (ER) or progesterone receptor (PR) for 60 minutes. After further washing in TBS, sections were incubated for 30 minutes with biotinylated secondary antibody, washed in TBS and incubated with ABC alkaline phosphatase (Vectastain alkaline phosphatase kit) for 30 minutes. The reaction was developed using alkaline phosphatase substrate kit III (Vecta Blue)(Vector). Sections were incubated at room temperature and the reaction was monitored microscopically until the appropriate blue colour developed after approximately 10 minutes. The reaction was stopped by excess water. Double-labelled slides were not counterstained.

Controls

Positive controls were as for single immunohistochemical labelling (above). Negative controls were performed for each tissue by replacement of both primary antibodies with normal serum. In addition for each double labelled section the primary antibody was omitted from each stage of the technique separately to produce the appropriate single immunolabelling. The different antibody-enzyme-substrate combinations were also reversed in the double labelled runs to confirm consistency of the reaction patterns with the different enzyme labels. The single labelled and double labelled slides were compared to confirm consistent distribution of labelling in each tissue and to check that no spurious double labelling had occurred.

Blocking Endogenous Peroxidase Activity

Endogenous peroxidase can cause non-specific staining with the peroxidase substrates DAB and AEC in immunohistochemistry. Routinely endogenous peroxidase activity is reduced by incubation with H_2O_2 in methanol at the start of the procedure. This technique was used for double labelled and paraffin sections.

Blocking of endogenous peroxidase with phenylhydrazine was evaluated for use in frozen endometrial biopsies in this study because of the finding in some negative controls of staining due to endogenous peroxidase, despite routine treatment with H_2O_2. This method has routinely been used in the Department of Pathology during the preparation for immunostaining of cytospin preparations of pleural and peritoneal fluid. The technique has been shown to be non-deleterious with respect to lymphocyte surface antigens (Andrew and Jasani 1987).

Blocking of endometrial endogenous peroxidase with phenylhydrazine was initially evaluated in frozen sections of tissue obtained from hysterectomy specimens from pre-menopausal women removed for non-endometrial pathology. It was tested at various stages in the immunohistochemical process and was considered to be most effective after the use of secondary antibody. There was no apparent effect on antibody binding due to cell surface damage or loss of surface antigens. Thus, following the application of biotinylated secondary antibody sections were washed in warm TBS for 10 minutes and then washed in a solution of 0.1% phenylhydrazine (Sigma Chemical Co.) at 37^0C for 45 minutes. The blocking step was followed by two 4-minute washes in TBS at room temperature and incubation with ABC reagent as previously described.

Mounting

Sections labelled with DAB were dehydrated in increasing alcohol concentrations followed by clearing in xylene. They were overlain with DPX (Raymond A. Lamb Ltd., London, UK) a synthetic resin mountant, and mounted with coverslips. Sections labelled with alcohol soluble products (AEC, Vecta Blue) were overlain with Supermount (Biogenex, San Ramon, USA), dried overnight in air and then permanently mounted with DPX and coverslips.

3. EVALUATION

All labelled sections were examined by eye under the microscope, either qualitatively for distribution of labelling or quantitatively using a 10x10mm graticule for individual cell counts.

Quantitative assessment

For quantitative assessment of immunohistochemical labelling (Chapter 5), sections were examined microscopically under high power (x40 objective and x10 eye-piece) and labelled cells counted directly using a 10x10mm graticule. The counts were expressed as a proportion of the total cell numbers in a given area.

Semi-quantitative assessment

For some antibody labelling a counting technique was not suitable either because of the overlapping of staining, its extracellular nature or a non-uniformity of labelling throughout a tissue. In these situations tissue areas under examination were given a score to indicate the proportion of cells or area labelled.

Staining Scores

0	=	no labelling
1	=	up to 20% labelling
2	=	20-40% labelling
3	=	40-60% labelling
4	=	60-80% labelling
5	=	80-100% labelling

Qualitative assessment

Double immunohistochemical labelling of sections was inspected for evidence of dual expression of the antigens examined as well as single labelling red or blue.

Statistical Analysis

The analysis of data presented in Chapters 3, 5 and 6, is complicated by the use of four subject groups sampled at two different times in the menstrual cycle. Multiple individual tests of significance would be likely to give a relatively high return of falsely significant results. In order to overcome this, analysis of variance (ANOVA) has been performed using Scheff's correction. The result of using such a technique is that it errs on the side of non-significance. The value of this however, is that any significant results are sound.

Since endometrial samples taken at LH+7 and LH+13 can be considered to be functionally distinct, in all evaluations apart from LH+7 versus LH+13, the data are examined as two separate sets of four subject groups. Comparison of samples at

LH+7 versus LH+13 employed the Mann-Whitney non-parametric test of significance. Mann-Whitney test was also used in Chapter 3 to compare serum oestrogen or progesterone concentrations from LH+7 and LH+13.

The paired endometrial samples examined in Chapter 5 were tested using 2-tailed, paired t-test.

In all cases $p<0.05$ denotes significance. Trends were inferred when $p<0.25$ or where changes in means or medians were all in the same direction in comparable groups.

Summary

The samples, materials and methods employed for the purposes of this thesis are described here. Variations from these techniques are discussed in the relevant chapters.

CHAPTER 3

CONTROL OF THE ENDOMETRIAL ENVIRONMENT

CONTROL OF THE ENDOMETRIAL ENVIRONMENT

1. STEROID HORMONE RECEPTOR DISTRIBUTION IN RELATION TO FERTILITY

Introduction

The complex relationships of the hypothalamo-pituitary-ovarian-endometrial axis have at their hub the steroid hormones oestrogen and progesterone, secreted in a cyclical fashion by the ovary in normally ovulating women. The pattern of secretion of these hormones is crucial to the overall cyclical performance of the endometrium and derangement of ovarian hormone secretion such as at the menopause or in anovulatory cycles has a profound effect on the pattern of menses; amenorrhoea or oligomenorrhoea. This relationship has allowed numerous endocrine manipulations to be used for a variety of purposes; including contraception and the control of menorrhagia amongst others.

The hormone/endometrium relationship has been well studied and is well documented in standard texts. Research has also focused on the possibility that fertility problems may be related to, for example, abnormal luteal function - so-called luteal phase deficiency (LPD). Evidence for the existence of this condition however, is variable. LPD was first described in 1949 by Georgianna Jones (Jones 1949) who considered urinary pregnanediol excretion and endometrial biopsies timed from and correlated with basal body temperature (BBT) changes. Although flawed from the point of view of timing and the accuracy of histological assessments, this study stimulated a vast area of research. Lenton et al. (1984) and Smith et al. (1984) focussing purely on cycle length, have shown that a short luteal phase is as likely to occur in fertile as in subfertile populations implying that if LPD plays a significant

role in fertility then it is not related to short cycles. Jordan et al. (1994) attempted to evaluate all the possible modes of assessment of the luteal phase, including BBT, follicular development, endometrial biopsy and progesterone production. They concluded that an assessment of serum progesterone concentrations (the sum of three measurements from LH+5 to LH+9) would establish a defective luteal phase if lower than 30ng/ml. This, however, implies a primary hormonal aetiology which should be correctable by the administration of progesterone. Rosenberg et al. (1980) and Soules et al. (1977) diagnosed LPD on the basis of late luteal phase endometrial biopsies from two cycles and treated with progesterone claiming conception rates of 71% and 50% respectively. Daly et al. (1983) used a variety of treatment regimes (progesterone, clomiphene citrate and gonadotrophins) for women when the endometrial maturity of biopsies taken on the 12^{th} post-ovulatory day were considered to be histologically delayed on two occasions. This group stressed the importance of "normalisation" of the endometrial biopsy and claimed an 81% conception rate in compliant patients where this had occurred. None of these studies provided non-treatment controls. Davidson et al. (1987), Wentz et al. (1990) and Balasch et al. (1986), however, in studies of endometrial biopsies showed that not only did treatment with progesterone or clomiphene citrate not improve the pregnancy outcome, but also that the incidence of biopsy diagnosed LPD was as common in the fertile as the subfertile population. It was concluded therefore, that the diagnosis of LPD was without clinical significance. There have been no randomised controlled trials of the treatment of LPD with progesterone to finally settle this argument.

It can be seen that a disorder of the luteal phase may have a variety of effects on the "window of implantation" alluded to previously (Chapter 1). A steroid hormone production problem has not been generally supported in the face of normal luteal phase progesterone concentrations in most ovulatory cycles, but there remains the possibility that the endometrial response to normal steroid hormone levels may be abnormal. This may result in an environment hostile to the process of implantation, leading to the inability to achieve or support a pregnancy which may then contribute to otherwise unexplained subfertility (Li and Cooke, 1991). The possibility of an abnormal endometrial response to endocrine stimuli has not previously been investigated and it could occur at a number of levels.

The expression of sex steroid receptors in human endometrium during the normal menstrual cycle has been well documented. Early studies using hormone-receptor binding techniques showed peaks of oestrogen and progesterone receptors in late proliferative and peri-ovulatory phases respectively, with a subsequent reduction in each through the secretory phase (Levy et al. 1980). This approach does not allow the study of individual cell types afforded by more recent immunohistochemical techniques (Press et al. 1986 and1988, Garcia et al. 1988, Lessey et al. 1988, Snijders et al. 1992, Coppens et al. 1993, Critchley et al. 1993, Amso et al. 1994). More specifically oestrogen receptors (ER) have been shown to be expressed in both epithelium and stroma in proliferative and secretory phase endometrium, peaking around ovulation. Immunostaining for ER in the mid and late secretory phase is reduced. Progesterone receptor (PR) expression similarly increases in glands and stroma through the proliferative phase, peaking around ovulation and in the early

secretory phase. Although stromal PR expression is relatively constant in both phases of the cycle, glandular expression generally falls to negative by the late luteal phase.

Although the general cyclical distribution of oestrogen and progesterone receptors has been described, there has been no previous examination of the receptor distributions in fertile compared with subfertile endometrium. The following study therefore was designed to examine the potential for normal and subfertile endometrium to respond to oestrogen and progesterone by assessment of their respective receptors in the endometrium of women with unexplained subfertility compared with normal controls.

Experimental Design

Endometrial samples

Pipelle endometrial samples obtained as previously described, from both fertile women and women with unexplained subfertility were studied from the subject groups described above (Chapter 2).

Monoclonal antibodies

Novocastra (Newcastle-upon-Tyne, UK) monoclonal antibodies (mAbs) to oestrogen receptors (ER-LH2) and progesterone receptors (PGR) were used at concentrations 1:20 and 1:100, respectively.

ER-LH2 and PGR are IgG class mouse antibodies raised for research purposes and producing nuclear staining patterns with immunohistochemistry. ER-LH2 is raised

against a recombinant oestrogen receptor fusion protein and PGR against a synthetic peptide corresponding to a site on the human progesterone receptor.

Single immunohistochemical labelling

Standard single immunohistochemical labelling with DAB was performed as described previously (Chapter 2) on frozen sections from both LH+7 and LH+13 samples obtained from the four subject groups.

Positive control was as described in Chapter 2. In addition, since the antibodies described had previously been fully evaluated for use in frozen sections of endometrium and decidua in this laboratory, endometrium provided an "in-built" positive confirmation. Negative controls, where no primary antibody was added contrasted with positive labelling in test sections and excluded significant non-specific staining.

Evaluation

All sections in this study were examined semi-quantitatively for oestrogen and progesterone receptor distribution. A score of 0 to 5 was used according to the proportion of cells positively labelled as described above (Chapter 2). The assessment was blind to the group and timing of the original sample.

Surface epithelium (when present), glandular epithelium and stroma were each scored separately in each section.

Statistical analysis

Statistical analysis of these data has been discussed in Chapter 2.

Results

The results reflect the general heterogeneity in expression of oestrogen and progesterone receptors in the secretory phase. There was a variation in staining between different sections as would be expected, but also in extent and intensity between areas of, for example, stroma in the same section, or from one gland to another or in different areas of the same surface epithelium.

LH+7 versus LH+13.

Expression of both receptor types was in general low at mid (LH+7) and late (LH+13) secretory phase (median scores ≤ 2 for expression of ER or PR in all groups), although no proliferative or early secretory phase endometrium was available from these subjects for direct comparison. The labelling of surface epithelium, identified in around 60% of sections examined, was patchy when present (Figs. 3.1a and 3.2a-b). Similarly glandular epithelial cells could all be labelled clearly in some glands whereas in other glands in the same specimen all the epithelial cells were negative (Figs. 3.1b-c and 3.2b-c). Stromal labelling was also generally variable in intensity (Figs. 3.1 and 3.2). Thus even the semi-quantitative scoring assessment had pitfalls, as it was difficult to represent an overall impression for each individual tissue.

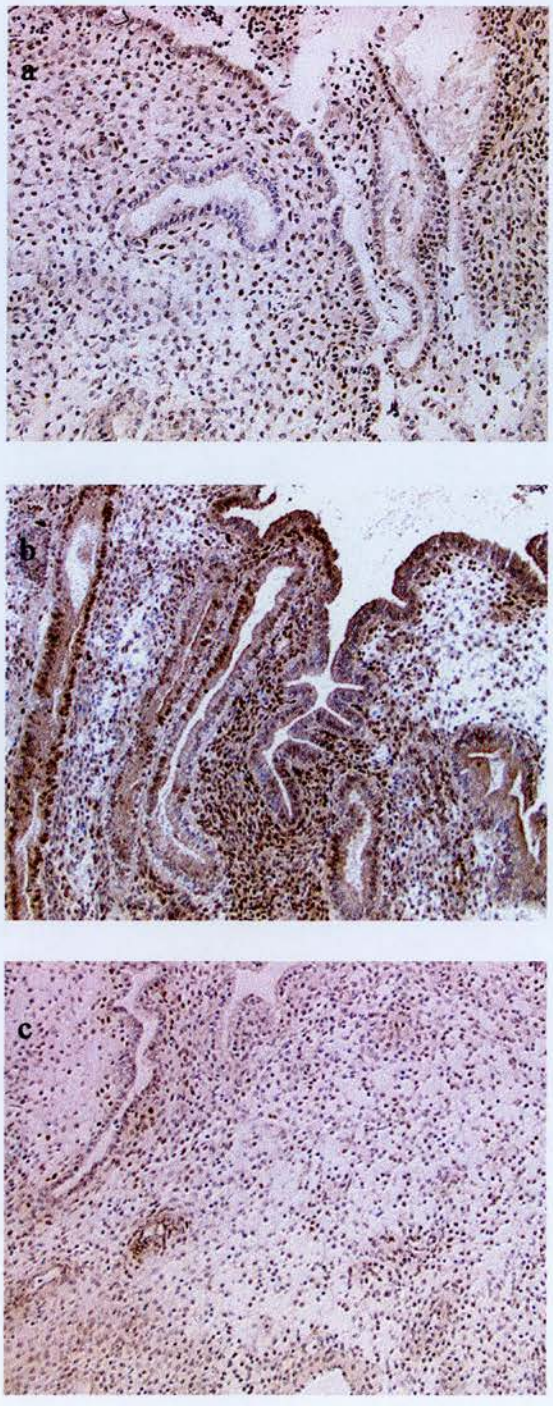


Figure 3.1. *Frozen sections of Pipelle endometrial samples. Single labelling, ER-LH2.* a) *LH+13 (x20);* b) *LH+7 (x10);* c) *LH+13 (x10).*

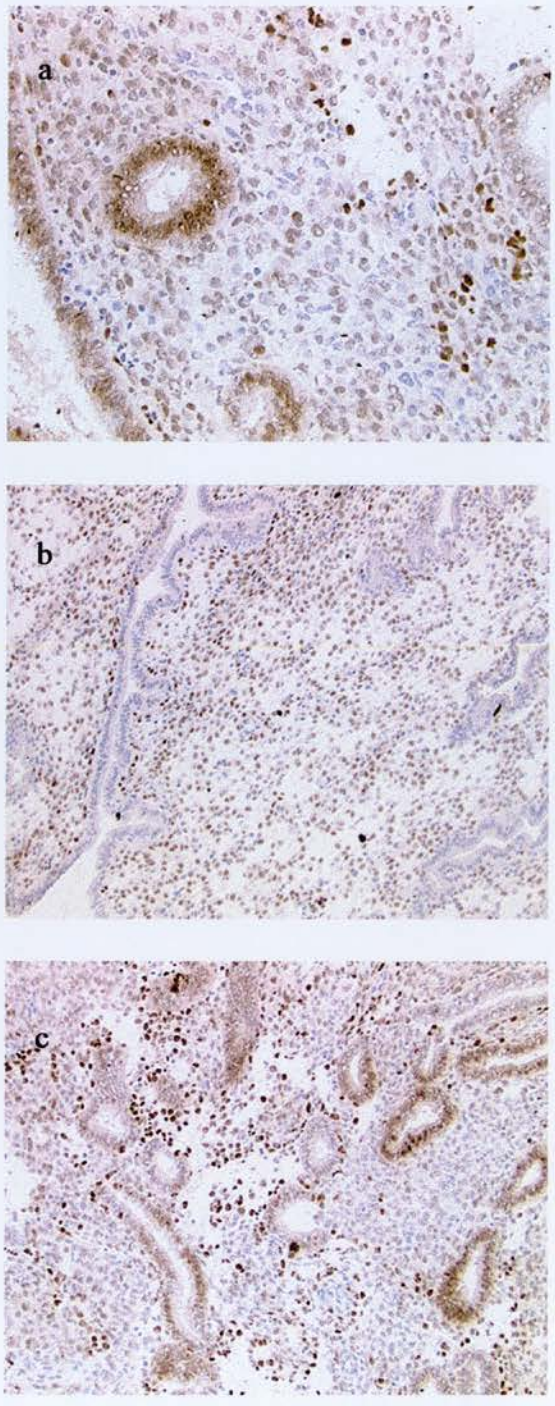


Figure 3.2. *Frozen sections of Pipelle endometrial samples. Single labelling, PGR* a) *LH+7 (x20);* b) *LH+13 (x10);* c) *LH+7 (x10).*

Oestrogen receptor labelling

Stromal labelling of ER, although showing a degree of variability within tissues, was in general similar in all tissues of each group and at both stages of the cycle examined. Most tissues scored 2 and all showed a degree of positive labelling. Glandular and surface epithelial staining was more heterogeneous both within tissues and between groups; occasional sections exhibited either no glandular epithelial or no surface epithelial labelling but with definite stromal labelling. Surface epithelium, because of the nature of Pipelle sampling and the subsequent tissue processing, was not identified in 38% of the sections examined.

There was no significant difference in ER expression between LH+7 and LH+13 (Table 3.1). When mean scores were considered there was a trend towards lower expression of oestrogen receptors in fertile glandular and particularly surface epithelial sites at LH+13 compared with LH+7. Of note however, is that this trend was apparently reversed in the endometrial epithelium of subfertile individuals (Fig. 3.3). In other words, in contrast with previous studies of normal endometrium, there was an increase in expression of epithelial oestrogen receptor from LH+7 to LH+13 in subfertile endometrium. It should be noted however, that for surface epithelium, sample numbers were low and the range of staining score wide, such that any such trend may be considered serendipitous.

Table 3.1. *Oestrogen receptor staining scores for fertile and subfertile endometrium. LH+7 versus LH+13.*

Oestrogen Receptors	**LH+7 staining score**				**LH+13 staining score**				**P**
Nulliparous Fertile	**n**	**median**	**mean**	**range**	**n**	**median**	**mean**	**range**	
Stroma	7	2	2.07	1-3	5	2	2.10	2-2.5	1.000
Glands	7	2	1.79	0-3.5	5	1	1.50	1-2.5	0.755
Surface Epithelium	4	2	2.25	1-4	2	0.5	0.50	0-1	*0.133*
Nulliparous Infertile									
Stroma	10	2	2.05	2-2.5	7	2	2.29	2-4	0.887
Glands	10	1	1.45	1-3.5	6	1	1.71	0-5	0.887
Surface Epithelium	9	2	1.78	0-3	5	2	1.92	0-4	0.776
Parous Fertile									
Stroma	8	2	2.50	2-4	9	2	2.06	2-2.5	0.321
Glands	8	2	2.38	1-5	9	2	1.56	0-2.5	0.370
Surface Epithelium	7	2	2.00	1-4	7	2	1.71	0-3	0.710
Parous Infertile									
Stroma	9	2	2.00	2-2	4	2	2.00	2-2	1.000
Glands	9	1	1.39	1-2.5	4	1	1.25	1-2	0.825
Surface Epithelium	7	2	1.71	0-3	1	2.5	2.50	2.5	0.500

Mann-Whitney. Reaches significance at $P \leq 0.05$.

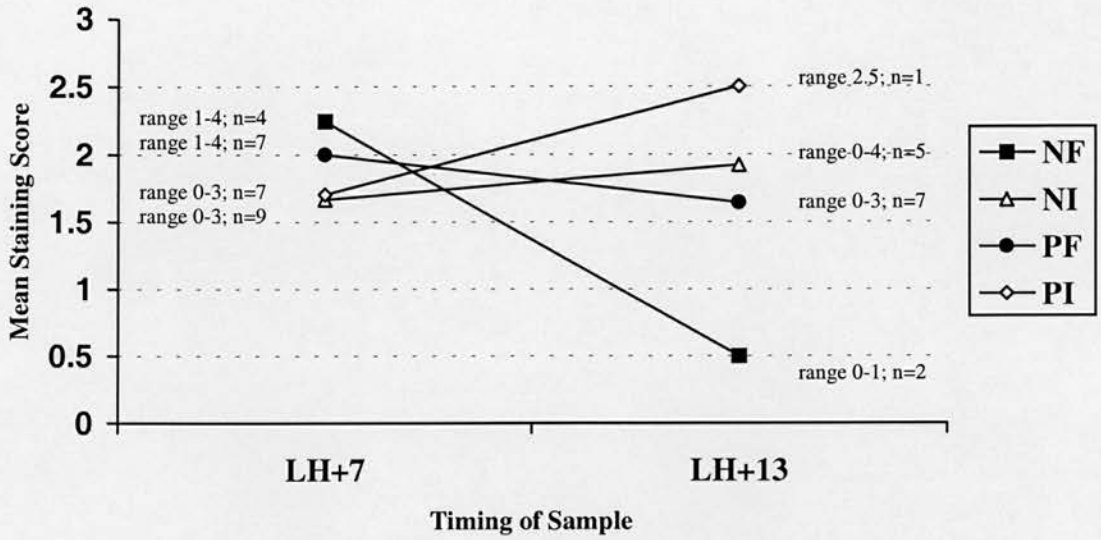


Figure 3.3. *The change in endometrial surface epithelial oestrogen receptor expression. LH+7 to LH+13.*

Progesterone receptor labelling

Stromal expression of PR was, like that for ER, fairly similar between samples and between groups (Table 3.2). Occasional tissues exhibited more marked labelling (scoring 3-5), but most again scored 2. PR labelling was mostly negative in both glandular and in surface epithelium. Where labelling was present it was more likely to be in glandular than surface epithelium. Occasionally more marked labelling occurred corresponding to high levels of labelling throughout the tissue. Again around 36% of sections examined did not contain identifiable surface epithelium.

Table 3.2. *Progesterone receptor staining scores for fertile and subfertile endometrium. LH+7 versus LH+13.*

Progesterone Receptors	**LH+7 staining score**				**LH+13 staining score**				**P**
Nulliparous Fertile	**n**	**median**	**mean**	**range**	**n**	**median**	**mean**	**range**	
Stroma	7	2	1.79	0-2.5	5	2	1.90	1-2.5	1.000
Glands	7	0	0.43	0-1	5	1	0.80	0-2	0.530
Surface Epithelium	5	0	0.80	0-2	4	0	0.50	0-2	0.730
Nulliparous Infertile									
Stroma	10	2	1.90	1-2.5	7	2	1.92	1-2.5	1.000
Glands	10	0	0.10	0-1	7	0	0.29	0-1	0.536
Surface Epithelium	8	0	0	0	6	0	0	0	1.000
Parous Fertile									
Stroma	8	2	2.44	1-5	9	2	2.44	2-5	0.815
Glands	8	1	1.63	0-4.5	9	0	0.83	0-4.5	*0.139*
Surface Epithelium	6	2	2.33	1-5	7	0	0.71	0-5	***0.022***
Parous Infertile									
Stroma	9	2	1.89	1-3	4	2	2.00	2	0.825
Glands	9	0	0.33	0-1	4	0.5	0.50	0-1	0.710
Surface Epithelium	6	0	0.50	0-2	2	0	0	0	0.643

Mann-Whitney. Reaches significance at $P \leq 0.05$.

It is of interest that there was only a very low level of expression of progesterone receptors in the surface epithelium in subfertile endometrium at both luteal phase stages assessed, whilst endometrium from fertile individuals exhibited a reduction in surface epithelial progesterone receptor expression from LH+7 to LH+13 (Fig.3.4). This reached significance in the parous fertile group ($p = 0.022$). The lack of this

reduction in subfertile endometrium may be explained by the reduced expression of PR at LH+7 compared with normal fertile controls.

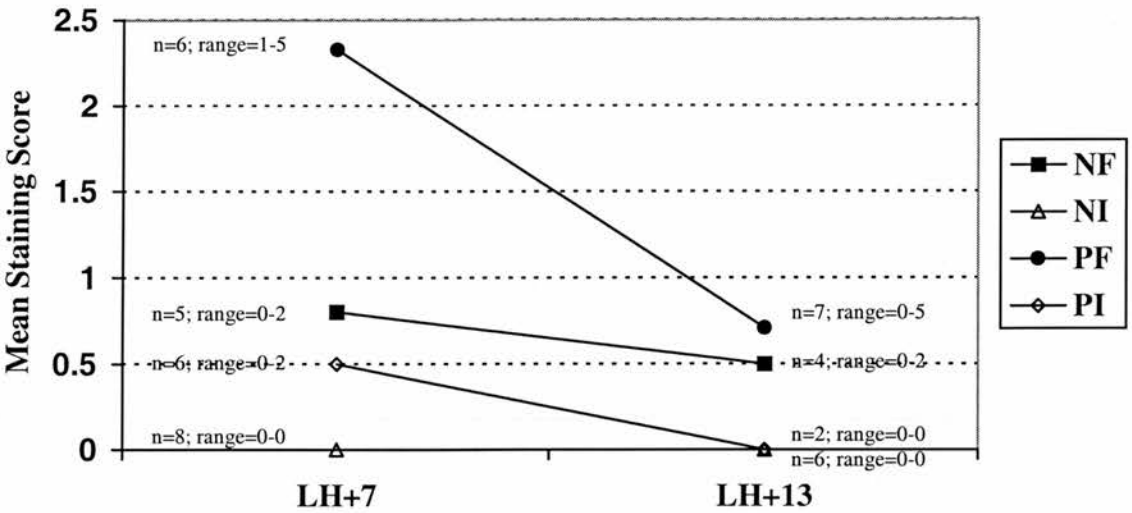


Figure 3.4. *The change in endometrial surface epithelial progesterone receptor expression. LH+7 to LH+13.*

Serum Oestrogen and Progesterone Concentrations

These women had carefully timed samples from the LH surge and in addition there were no significant differences between the four patient subgroups in serum progesterone and oestradiol concentrations at the time of sampling (Figs. 3.5 and 3.6).

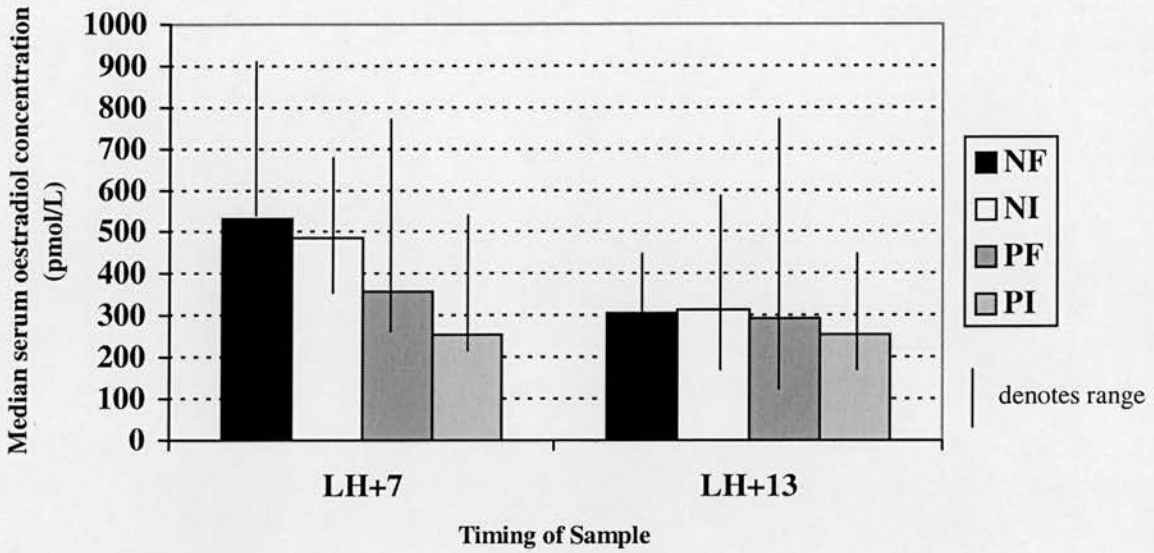


Figure 3.5. *Serum oestradiol concentrations. LH7 to LH13.*

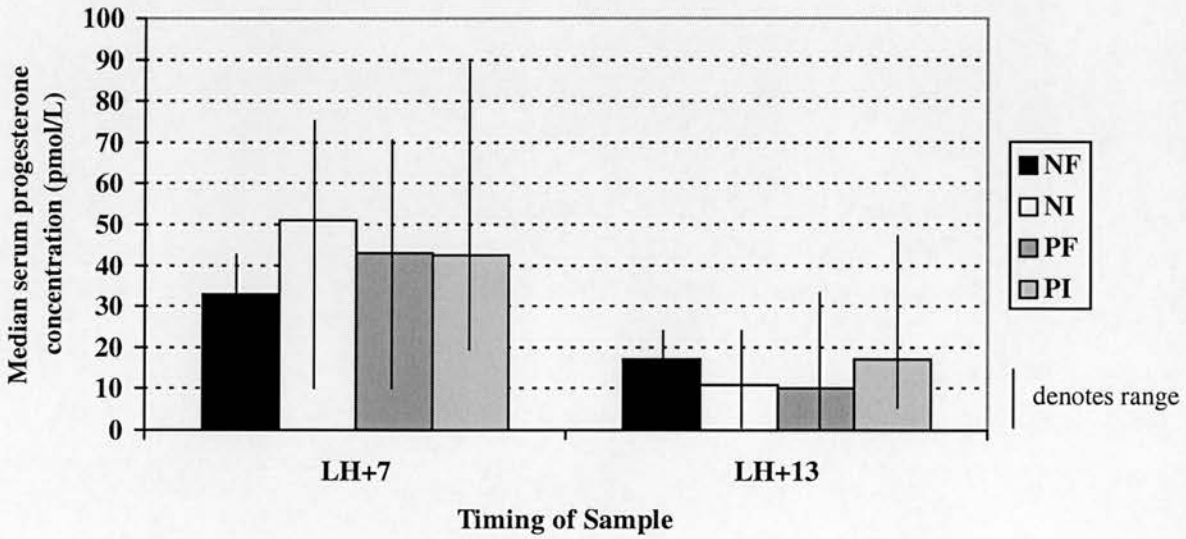


Figure 3.6. *Serum progesterone concentrations. LH7 to LH13.*

The Effect of Previous Parity and Fertility Status.

When endometrium from the four subject groups was compared at LH+7 and at LH+13 there were few clear differences in oestrogen receptor distribution between fertile and subfertile and between parous and nulliparous individuals (Fig. 3.7). Interestingly however, there were significant differences in the progesterone receptor distribution at LH+7; there was less epithelial progesterone receptor expression in subfertile compared with fertile endometrium whether nulliparous or parous, reaching significance in the parous group at LH+7 (Table 3.3). These findings are in keeping with the idea of an earlier loss of epithelial progesterone receptor expression in subfertile compared with fertile endometrium. In addition it was found that expression was greater in parous as compared to nulliparous endometrium, again approaching significance at LH+7.

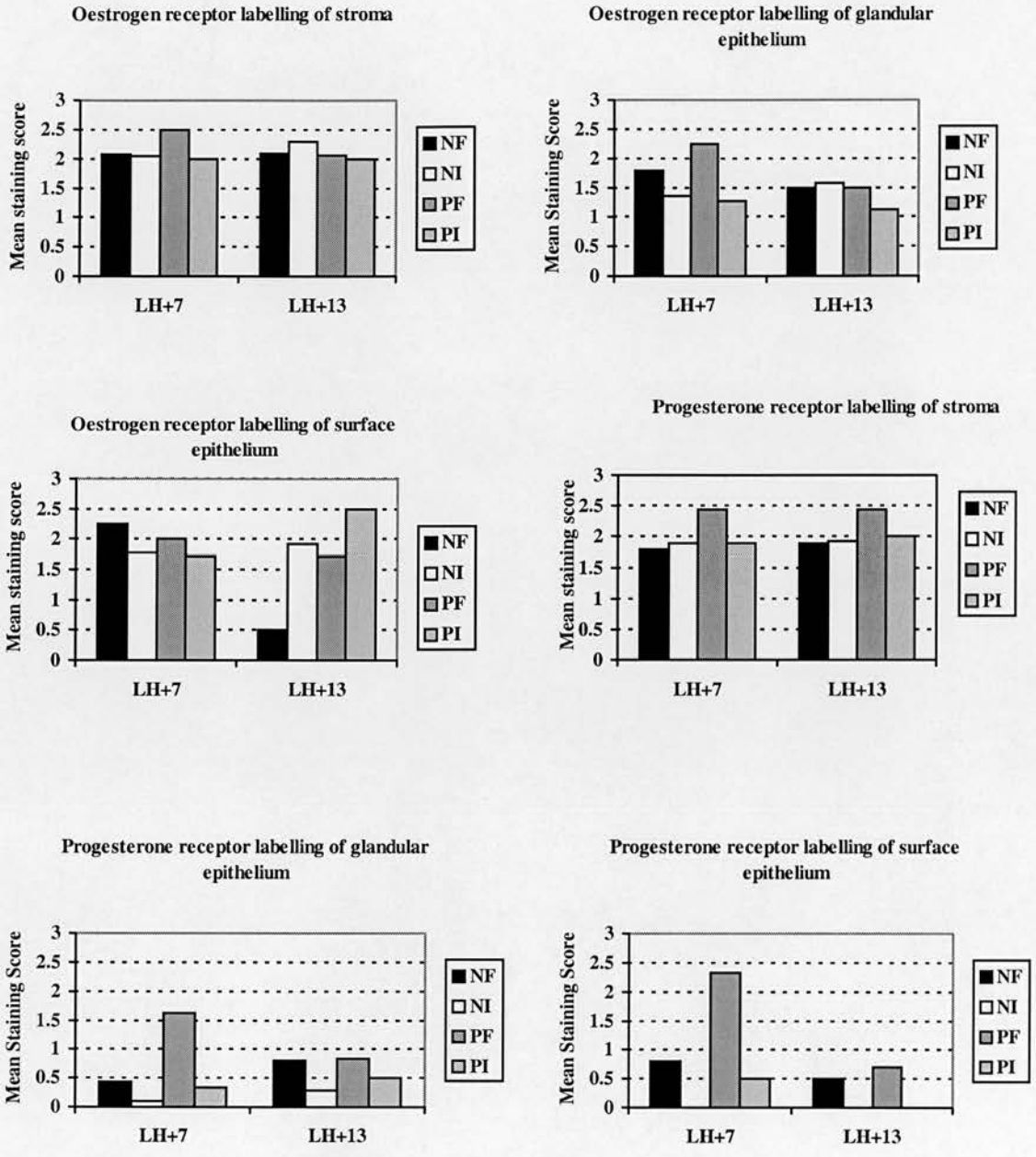


Figure 3.7. *Oestrogen and progesterone receptor expression in endometrial stroma, glandular epithelium and surface epithelium and taking into account fertility and parity.*

Table 3.3. *Oestrogen and progesterone receptor distributions. Significances when comparing fertile versus subfertile and parous versus nulliparous endometrium.*

	Nulliparous	**Parous**	**Fertile**	**Subfertile**
	Fertile vs. subfertile	Fertile vs. subfertile	Parous vs. nulliparous	Parous vs. nulliparous
LH+7	Significance (p)			
Oestrogen Receptors				
Stroma	1.000	*0.200*	0.379	0.997
Glands	0.853	0.282	0.848	0.999
Surface Epithelium	0.838	0.967	0.986	1.000
Progesterone Receptors				
Stroma	0.995	0.640	0.555	1.000
Glands	0.887	***0.031***	*0.075*	0.946
Surface Epithelium	0.561	***0.031***	*0.108*	0.821
LH+13				
Oestrogen Receptors				
Stroma	0.907	0.997	0.998	0.770
Glands	1.00	0.948	1.000	0.925
Surface Epithelium	0.532	0.695	0.440	0.788
Progesterone Receptors				
Stroma	1.00	0.770	0.587	0.999
Glands	0.865	0.961	1.000	0.990
Surface Epithelium	0.945	0.920	0.995	1.000

n, medians, means and ranges for each group have been recorded in Tables 3.1 and 3.2.

2. BCL-2 AND KI67 EXPRESSION IN ENDOMETRIUM IN RELATION TO FERTILITY.

Introduction

Bcl-2 is an oncoprotein encoded by the *bcl*-2 (B cell lymphoma/leukaemia-2) oncogene. It is known to protect cells against apoptosis, although the exact mechanism of action has not been elucidated (Reed 1994, Brown 1996). Several studies have examined bcl-2 expression in human endometrium and it appears to be generally agreed that major expression of bcl-2 is in glandular epithelium, peaking at the end of the proliferative phase. Expression of bcl-2 in endometrial epithelial cells has been reported to decrease (Otsuki et al. 1994; Tabibzadeh et al. 1995) or be absent (Gompel et al. 1994) in the secretory phase of the cycle, although Koh et al. (1995) reported an increase in bcl-2 expression in the late secretory phase at these sites. Stromal bcl-2 expression also displays a degree of cyclical variation increasing during the secretory phase (Gompel et al. 1994, Koh et al. 1995, Tabibzadeh et al. 1995).

The increase in stromal bcl-2 expression in the late secretory phase of the menstrual cycle has been attributed by some to the large numbers of eGLs present (Chapter 4), but expression of bcl-2 on stromal cells (and on other leucocyte groups) remains a possibility. Although the function of bcl-2 in endometrium is not clear, it has been suggested (Koh et al. 1995) that glandular and stromal expression have separate roles in the maintenance of endometrial surface integrity and in the control of onset of menstruation, respectively. However, bcl-2 protects cells from apoptosis and the findings by Koh et al. (1995) of increased bcl-2 expression in the late secretory phase are not consistent with the reports of increased apoptosis in glandular epithelium at

this time (Tabibzadeh 1994). It is possible that the protection against apoptosis afforded by expression of bcl-2 antigen is incomplete. This could be inferred from the work of Tabibzadeh et al. (1995) who suggested that secretion of TNFα, increased throughout the menstrual cycle in relation to a reduction in bcl-2 endometrial epithelial expression. It should be noted that bcl-2 is only one of a family of proteins involved in cell longevity and in addition is probably dependent on bound ligands; for example, the bax protein for normal function (Brown 1996). Thus until the mechanism of action of bcl-2 and its related proteins are established its significance in any situation must be somewhat speculative.

Ki67, a cell proliferation marker, is known to be expressed in endometrial stroma to different degrees throughout the menstrual cycle. Expression of Ki67 is high in endometrial stroma in the proliferative and early secretory phases, decreasing in the mid-secretory phase but increasing again premenstrually (Jones et al. 1998a). This final increase corresponds to the proliferation of eGLs in the secretory phase of the cycle and Ki67 has been shown by single and double immunohistochemical techniques to be expressed by these cells (Pace et al. 1989, King et al. 1991, Jones et al. 1998a). Endometrial glandular epithelial cells have been shown, as expected, to be strongly labelled for Ki67 in the proliferative phase (Pace et al. 1989, Tabibzadeh 1990a). Ki67 labelled cells have been reported to remain numerous in the epithelium in the secretory phase also, especially in the early secretory phase (Pace et al. 1989), although Tabibzadeh (1990a) found markedly diminished glandular epithelial proliferation in early secretory phase endometrium and none in mid and late secretory phases.

The expression of bcl-2 and Ki67 have not previously been investigated in subfertile endometrium. However, as the turnover of cells in the endometrium is likely to be of great importance to the maintenance of its normal structure and consequently its function, altered expression of either of these antigens may indicate a problem relating to subfertility. The second study in this section therefore, was designed to examine the expression of bcl-2 and Ki67 in the endometrium during the secretory phase of the menstrual cycle, taking into account both previous parity and current fertility status.

Experimental design

Endometrial samples

For this study sections of frozen Pipelle endometrial samples from subjects recruited and sampled as discussed previously (Chapter 2) were used.

Antibodies

(See Table 3.4)

Table 3.4. *Monoclonal Antibodies*

Antibody	**Specificity**	**Source**	**Concentration**
Bcl-2	Proto-oncogene product	Novocastra	1:100
Ki67	Proliferation Marker	Novocastra	1:10

Bcl-2 antibody, raised against a synthetic peptide sequence, was first evaluated on similarly processed control tissues and a working concentration of 1:100 established as the optimum for this study. Ki67 had previously been evaluated for use in this lab.

Immunohistochemistry

Single immunohistochemical staining was performed in all sections as described in Chapter 2. DAB was used as the peroxidase substrate giving a brown reaction product. Since expression of both bcl-2 and Ki67 in endometrium has been previously described in detail, endometrium acted as its own in-built positive control. Negative controls where no primary antibody was used were included in all experiments to exclude non-specific labelling.

Evaluation

Stromal Ki67 and bcl-2 labelling was examined quantitatively. The proportion of stromal cells expressing bcl-2 or Ki67 was assessed by direct counting from 2 high-power fields of each section using a 10x10mm graticule. Around 1000 stromal cells per section were counted. The number of labelled cells was expressed as a percentage of the total number of stromal cells. The general distribution of Ki67 and bcl-2 expression in glandular and surface epithelial cells was also examined qualitatively in each section. The extent of labelling in these epithelial cells was given a score (0 to 5) relating to the proportion of cells stained as described above (Chapter 2).

Statistical analysis

Details of statistical analysis of these data have been discussed (Chapter 2).

Results

Bcl-2

Stromal bcl-2 expression in both subfertile and fertile endometrium appeared to increase from LH+7 to LH+13 (Table 3.5) (Figs.3.8 and 3.9), but this increase only approached significance in the nulliparous fertile group (P = 0.073).

There were no significant differences between the expression in fertile and subfertile or nulliparous and parous endometrium at either stage of the cycle examined.

Table3.5. *Endometrial bcl-2 expression. LH+7 and LH+13.*

		LH+7			**LH+13**		
		Stroma[1]	**Glandular epithelium[2]**	**Surface epi-thelium[2]**	**Stroma[1]**	**Glandular epithelium[2]**	**Surface epi-thelium[2]**
NF	N	7	7	7	5	5	5
	Mean	2.18	1.14	1.71	4.70	1.5	3.4
	Median	1.56	1	1	3.32	2	4
	Range	0.63-5.49	0-3	0-3.5	2.01-10.93	0.5-2	2-4
NI	N	12	12	12	8	8	8
	Mean	2.25	0.75	1.88	3.66	1.56	2.88
	Median	1.44	0.5	2	3.13	1.25	3.5
	Range	0.48-7.08	0-3	0-4	0.79-9.95	0-2.5	0-4
PF	N	9	9	9	9	9	9
	Mean	2.21	0.89	2.22	3.93	2.11	3.44
	Median	2.26	0	2	2.68	2	4
	Range	1.06-2.98	0-4	0-4	1.47-13.34	1-3	2-4
PI	N	10	10	10	5	5	5
	Mean	2.52	0.95	1.95	2.68	1.4	3
	Median	1.72	1	2	2.66	1.5	3
	Range	0.89-7.25	0.5-1.5	1-3	0.36-5.13	0-2.5	1-4

[1] % total stromal cells labelled with bcl-2 antibody. [2] staining score.

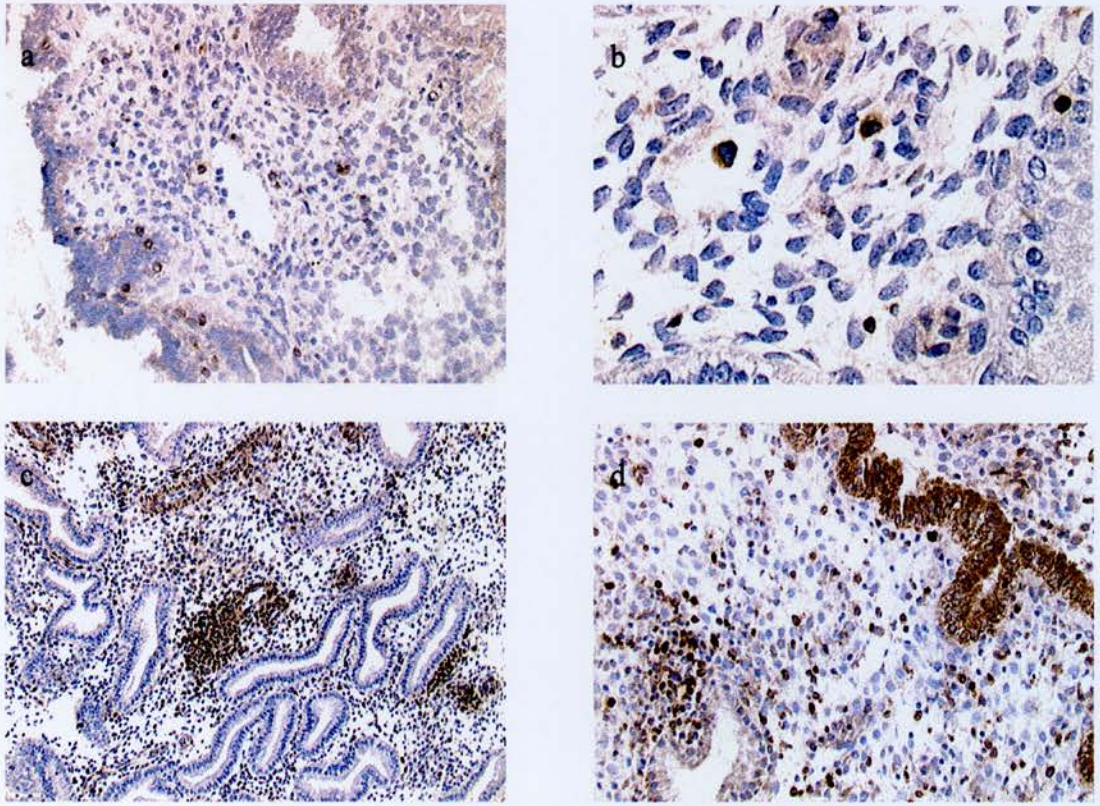


Figure 3.8. *Frozen sections of Pipelle endometrial samples. Bcl2 labelling.* a) *infertile, LH+7 (x20);* b) *infertile, LH+7 (x40);* c) *infertile, LH+13 (x10);* d) *fertile, LH+13 (x20).*

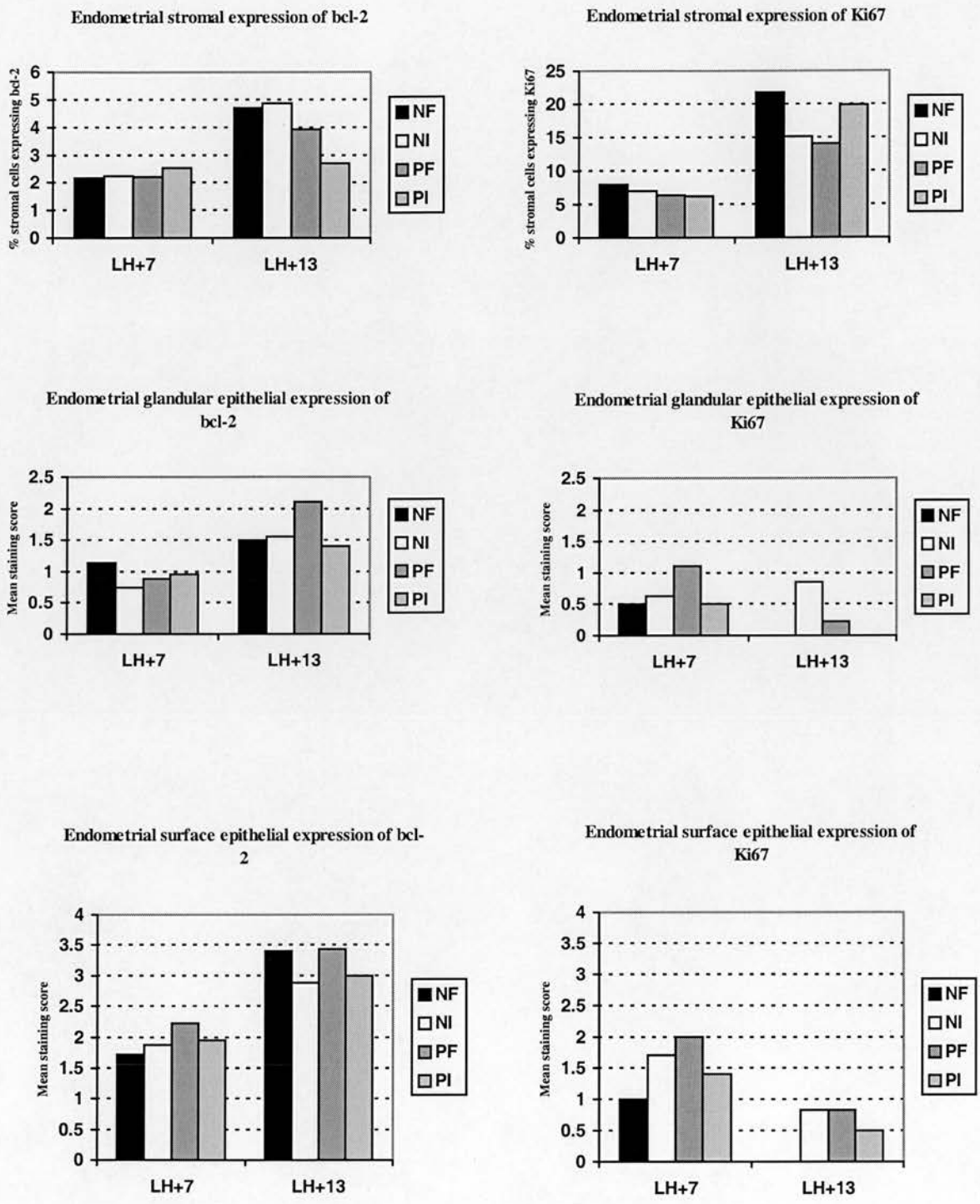


Figure 3. 9. *Endometrial expression of bcl-2 and Ki67.*

Semi-quantitative evaluation of glandular and surface epithelial expression of bcl-2 showed an overall increased expression of bcl-2 at LH+13 compared with LH+7. This was generally significant in some fertile groups (glandular epithelium; PF; p=0.019,surface epithelium; NF; p=0.048), and excluding NF glandular epithelium (p=0.53) approached significance in all other cases (Table 3.6).

When the effect of parity was considered or fertile and subfertile groups were compared there were no clear differences in expression of bcl-2 in endometrial glandular or surface epithelium.

Table 3.6. *Endometrial epithelial expression of bcl-2. LH+7 versus LH+13.*

	LH+7 vs. LH+13. Significance (p)
Glandular Epithelium	
Nulliparous Fertile	0.53
Parous Fertile	***0.019***
Nulliparous Infertile	*0.181*
Parous Infertile	*0.165*
Surface Epithelium	
Nulliparous Fertile	***0.048***
Parous Fertile	*0.063*
Nulliparous Infertile	*0.157*
Parous Infertile	*0.099*

Mann-Whitney. Reaches significance when $P \leq 0.05$.

Ki67

Stromal Ki67 expression was significantly greater at LH+13 than LH+7 in all groups (Figs.3.9 and 3.10; Tables 3.7 and 3.8).

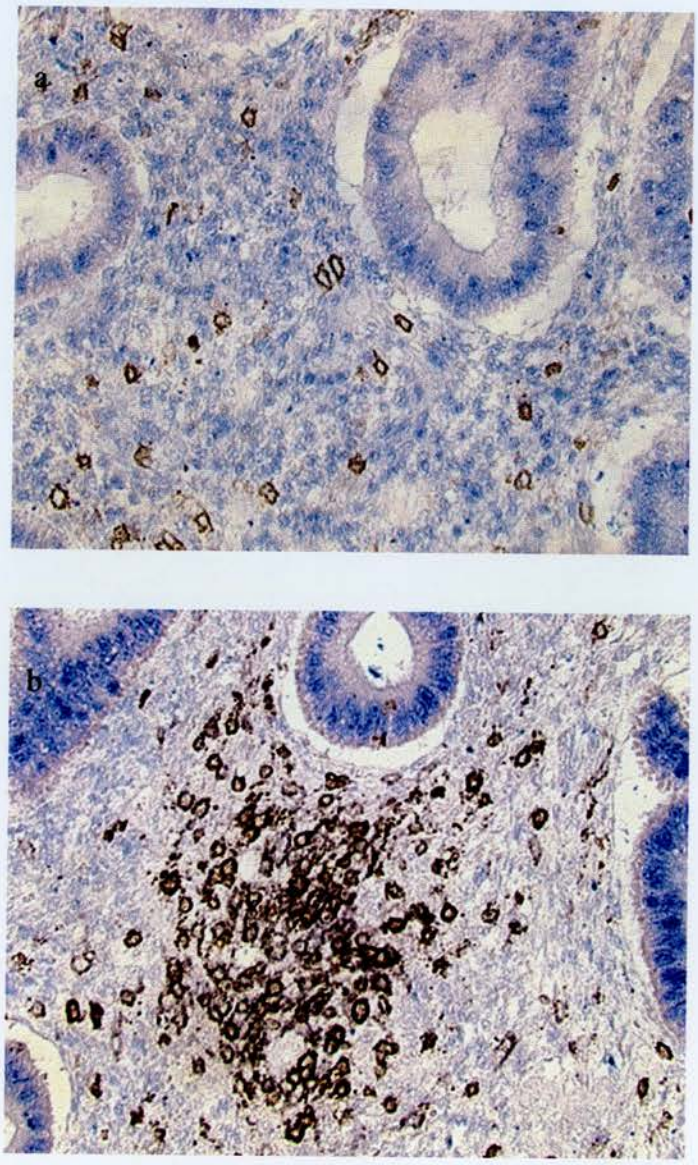


Figure 4.1. *Paraffin wax sections of secretory endometrium. Double labelled;CD45 (red), ER-6F11 (blue).* a) *(x20);* b) *(x20).*

Table 3.7. *Endometrial Ki67 expression. LH+7 and LH+13.*

		LH+7			LH+13		
		Stroma[1]	**Glandular epithelium[2]**	**Surface epi-thelium[2]**	**Stroma[1]**	**Glandular epithelium[2]**	**Surface epi-thelium[2]**
NF	n	6	6	6	4	4	1
	Mean	7.92	0.5	1	21.77	0	0
	Median	6.40	0	1	23.64	0	0
	range	3.90-19.26	0-2	0-2	14.91-24.87	0-0	0
NI	n	12	11	10	7	7	6
	Mean	6.98	0.64	1.7	15.12	0.86	0.83
	Median	5.90	0	2	11.41	1	0.6
	range	3.28-14.63	0-3	0-4	4.61-24.76	0-3	0-3
PF	n	9	9	7	9	9	6
	Mean	6.42	1.11	2	14.07	0.22	0.83
	Median	5.66	1	2	14.99	0	1
	range	2.88-17.79	0-3	0-4	4.88-22.64	0-2	0-2
PI	n	8	8	5	5	5	4
	Mean	6.24	0.5	1.4	19.90	0	0.5
	Median	5.55	0	1	21.24	0	0.5
	range	2.54-11.49	0-2	1-2	4.04-33.29	0-0	0-1

[1] % total stromal cells labelled with bcl-2 antibody. [2] staining score.

Table 3.8. *Endometrial stromal expression of Ki67. LH+7 versus LH+13.*

	LH+7 vs. LH+13. Significance (p)
Nulliparous Fertile	***0.019***
Parous Fertile	***0.014***
Nulliparous Infertile	***0.028***
Parous Infertile	***0.045***

Mann-Whitney. Reaches significance when $P \leq 0.05$.

In contrast there was a trend to reduced expression of Ki67 in endometrial epithelial sites at LH+13 compared with LH+7 (Figs.3.9 and 3.10). This was particularly of note in surface epithelium where lower expression approached significance in most groups (Table 3.9). There was no significant difference in Ki67 expression between

fertile and subfertile endometrium in the stroma or in surface or glandular epithelium. Similarly parity in either fertile or subfertile groups at LH+7 or LH+13 did not appear to affect Ki67 expression in stroma or epithelium.

Table 3.9. *Endometrial epithelial expression of Ki67. LH+7 versus LH+13.*

	LH+7 vs. LH+13. Significance (p)
Glandular Epithelium	
Nulliparous Fertile	0.476
Parous Fertile	*0.063*
Nulliparous Infertile	0.659
Parous Infertile	0.524
Surface Epithelium	
Nulliparous Fertile	0.286
Parous Fertile	*0.138*
Nulliparous Infertile	*0.118*
Parous Infertile	*0.111*

Mann-Whitney. Reaches significance when $P \leq 0.05$.

Discussion

The steroid hormones oestrogen and progesterone, play a central role in the cyclical changes occurring throughout the menstrual cycle in the endometrium. These hormones act via receptors which have previously been examined quantitatively from using receptor binding techniques in preparations of cytoplasm and nuclei from endometrial biopsies (Levy et al. 1980). The development of monoclonal antibodies and their continual improvement now allows receptor distribution to be examined in detail using an immunohistochemical approach.

The distribution of oestrogen and progesterone receptors in normal endometrium is well documented (Press et al. 1986, 1988; Garcia et al. 1988; Lessey et al. 1988; Snijders et al. 1992; Coppens et al. 1993; Critchley et al. 1993; Amso et al. 1994) and this study has confirmed results of previous studies in normal endometrium. However, a potential difference in the pattern of distribution of oestrogen and progesterone receptors in women with unexplained subfertility compared with the fertile population has been observed. Whilst stromal expression remained relatively constant, there was a tendency not to lose epithelial oestrogen receptor expression at LH+13 as was shown to occur in normal endometrium, despite similar serum concentrations of the hormone. This was reflected in the finding that there was generally lower expression of both oestrogen and progesterone receptor in the subfertile endometrium, both parous and nulliparous, except for epithelial oestrogen receptors at LH+13 which appeared to be relatively increased. This may simply be an example of the heterogeneity displayed by endometrial tissues at these stages in the menstrual cycle. However, these observations could be of significance with respect to the endometrial response to hormones and their secondary functions either as a consequence of, or resulting in an aberrant tissue response to steroid hormone stimuli. This would clearly be of importance with regard to fertility. In addition, subfertile endometrial epithelial tissue appeared to exhibit an earlier loss of progesterone receptor expression compared with fertile endometrium. This, in the face of similar serum hormone concentrations, may represent a functional difference between the groups and be associated with infertility.

Endometrial bcl-2 expression assessed in this study was generally in keeping with the distribution reported by Koh et al. (1995). In the secretory phase there was variable stromal expression (although there are no related proliferative Pipelle samples with which to compare) and no significant change between LH+7 and LH+13. There was however, an increase in epithelial and glandular bcl-2 expression at LH+13 compared with LH+7 in both fertile and subfertile samples.

The assessment of stromal Ki67 expression in endometrium was in keeping with previous studies (Jones et al. 1998a). Although the mid-secretory reduction in expression was not demonstrated, this study importantly confirms a high level of expression of this proliferation antigen pre-menstrually. The reduction in epithelial expression of Ki67 as the cycle progresses may be expected as menstruation is approached. This is in keeping with the findings of Pace et al. (1989) where maximal epithelial Ki67 expression was found in early secretory phase endometrium. Cessation or reduction of active proliferation would suggest reduced activity of the glandular and surface epithelial cells moving into the mid-secretory phase. Perhaps the increase in bcl-2 expression helps to maintain the viability of these otherwise aging cells which are not being so regularly replaced until such time as the onset of menstruation.

Conclusion

This study has supported the finding that Ki67 expression increases significantly premenstrually and since this has been previously shown to co-localise to leucocytes probably confirms that this activation is consistent irrespective of fertility status or

previous parity. There was a similar pattern of epithelial and stromal bcl-2 expression in all groups also suggesting that the cell dynamics of the secretory phase may not be fundamental to the problem of unexplained subfertility. However, there appeared to be important differences in oestrogen and progesterone receptor distribution in the luteal phase endometrium of women with unexplained subfertility which could have a fundamental effect on the tissue responses to those hormones.

CHAPTER 4

THE CONTROL OF ENDOMETRIAL LEUCOCYTE POPULATIONS

THE CONTROL OF ENDOMETRIAL LEUCOCYTE POPULATIONS

Introduction

As discussed previously (Chapter 3) the endometrium is a steroid sensitive organ, which responds to oestrogen and progesterone in a cyclical fashion. The morphological response during the different cycle phases has been well documented (Noyes et al. 1950, Good and Moyer 1968, Li et al. 1988, March 1991). The stromal leucocyte populations in human endometrium have also been reported to change during the menstrual cycle (King et al. 1989, Bulmer et al. 1991b, Starkey et al. 1991). In particular endometrial granulated lymphocytes (eGLs) increase in number dramatically in the late secretory phase of the menstrual cycle; if conception and implantation occur eGLs continue to increase in number and comprise around 70% of the stromal leucocytes in first trimester decidua (Bulmer et al. 1991b, Klentzeris et al. 1992) (see Chapter 1).

The control of the alterations in eGL numbers during the menstrual cycle remains poorly understood. The increase in numbers of CD56 positive eGLs has been postulated to be either the result of margination of peripheral blood NK cells or to be due to proliferation of cells in situ within the endometrium. Evidence for eGL proliferation comes from studies by Klentzeris et al. (1992) examining their morphometry. They concluded that increasing eGL numbers from LH+4 to LH+13 without change in the total fraction of endometrium occupied by eGL nuclei was indicative of local proliferation rather than recruitment. Further evidence comes from the work of Pace et al. (1989) and Tabibzadeh (1990) who investigated, using immunohistochemical techniques, the expression of proliferation associated antigens,

namely Ki67, on these cells as previously discussed (Chapter1). Although there is evidence allowing the possibility of recruitment of cells into the endometrium from the vasculature (Jones et al. 1997), proliferation of eGLs has been the generally accepted mechanism for the majority of their increase in numbers.

The reduction of CD56 positive eGLs after menstruation could be the result purely of menstrual flow, requiring regeneration of numbers in the subsequent menstrual cycle. The presence of eGLs in the stratum basalis of the endometrium supports this possibility providing reserve cells to allow for regeneration. It has been postulated by others, however, that eGL numbers may decrease in a non-pregnancy menstrual cycle as the result of apoptosis, presumably in a hormone dependent way. The unusual morphology of the cells in the premenstrual endometrium has lent weight to this theory (Loke and King 1985, King et al. 1989) (see below). The overall control of eGL numbers remains to be discovered but it seems likely that the primary factors will be steroid hormones which have such major roles in other glandular and stromal structural changes in endometrium throughout the menstrual cycle.

It appears likely that eGLs respond to the sex steroid hormones which influence the menstrual cycle, namely oestrogen and progesterone. This could be a direct effect on the leucocytes, which must in consequence express the appropriate receptors, or an indirect effect mediated by products of other endometrial cells which themselves express steroid hormone receptors. Direct stimulation of leucocytes by sex steroids has been suggested by reports that in pregnancy peripheral blood CD8 positive T lymphocytes express progesterone receptors (Szekeres-Bartho et al. 1990). Earlier

binding studies, however, excluded the expression of androgen, oestrogen and progesterone receptors on peripheral blood lymphocytes (PBL), including those activated by phytohaemagglutinin (PHA) (Neifeld et al. 1977). Progesterone has been reported to inhibit lymphocyte proliferation. This action, however, was not blocked by RU486, a progesterone (and glucocorticoid) antagonist, suggesting a mechanism not related to direct stimulation of progesterone receptors (Monterroso et al. 1993). Immunohistochemical studies of leucocytes in non-pregnant human endometrium have detected oestrogen receptor but not progesterone receptor expression by T cells in aggregates adjacent to endometrial glands in the basal endometrium (Tabibzadeh and Satyaswaroop 1989). However, King et al. (1996) reported conflicting results, having shown no evidence of any sex steroid hormone receptor expression by endometrial leucocytes. The latter study was confined to the general leucocyte population (CD45 positive), without examination of the specific leucocyte subgroups. Inoue et al. (1996) cultured endometrial leucocytes in the presence of progesterone, reporting a significant increase in the CD56 positive population after 6 days. They concluded that progesterone was important for in eGL proliferation or differentiation. Their cultures were leucocyte rich co-cultures with endometrial stromal cells however; and thus an indirect effect of progesterone via stromal cells and secondary messengers was not excluded.

This study was designed to assess any direct influence of steroid hormones on endometrial leucocyte populations, in particular on the proliferation of CD56-positive eGLs. In addition the role of apoptosis in cell demise at menstruation was considered.

1. Steroid Hormone Receptor Expression By Endometrial Leucocytes.

Materials and Methods

Frozen tissue

Fresh endometrial tissue was obtained from 15 hysterectomy specimens (5 proliferative, 5 early secretory, 5 late secretory) where the operation was performed for non-endometrial pathology such as fibroids not involving the uterine cavity, benign ovarian cysts or persistent cervical intraepithelial neoplasia (CIN). All the specimens had normal endometrial histology in keeping with the menstrual dates. Tissues were frozen and sections prepared as described above (Chapter 2). Decidual samples from 5 first trimester suction terminations of pregnancy were obtained with informed consent of the subjects. Again cryostat sections were prepared as described in Chapter 2.

Paraffin embedded sections

Sections (3μm) of late secretory phase endometrium (n=5) and normal first trimester decidua (n=5) from similar patient groups to those described above, were cut from archival paraffin blocks. Late secretory phase endometrium and decidua were used since maximal changes in endometrial leucocyte numbers occur at these stages (Chapter 1).

Monoclonal antibodies

Antibody specificities, dilutions and sources are given in Table 4.1. ER-LH2 and PGR were used to identify the distribution of oestrogen and progesterone receptors by immunohistochemical labelling in both frozen and paraffin sections. ER-LH2

was less satisfactory when used on paraffin-embedded tissues and therefore was substituted by ER-6F11 which gave good quality staining in paraffin-embedded sections after high temperature antigen retrieval (Chapter 2).

Leucocyte populations were identified by single and double immunohistochemical staining procedures in both frozen and paraffin sections using antibodies to CD45 (leucocyte common antigen), CD3 (T cells) and CD14 (macrophages). Endometrial granulated lymphocytes were identified using an antibody to CD56 (Novocastra), which has been well established in this laboratory for use on frozen sections. In paraffin-embedded sections eGL distribution was confirmed using MT-1 antibody (Bulmer et al. 1987) and an anti-CD56 antibody from Vector, Peterborough, UK (Table 4.1).

Table 4.1. *Monoclonal antibodies.*

	SPECIFICITY	**SOURCE**	**DILUTION**	
			Frozen sections	**Paraffin sections**
ER-LH2	Oestrogen receptor	Novocastra, Newcastle-upon-Tyne, UK.	1/30	1/10
ER-6F11	Oestrogen receptor	Novocastra		1/10
PR	Progesterone receptor	Novocastra	1/100	1/100
CD3	T lymphocytes	Dako, High Wycombe, UK.	1/200	
CD3	T lymphocytes	Novocastra		1/100
CD14	Macrophages	Dako	1/20	
CD45	Leucocyte common antigen	Dako	1/200	1/200
CD56	NK cells	Novocastra	1/200	
CD56	NK cells	Vector, Peterborough, UK		1/100
MT-1	(CD43) NK cells, T cells	Novocastra		1/40

Immunohistochemical labelling procedures

A. SINGLE LABELLING.

Single immunohistochemical labelling was performed as described previously (Chapter 2) using the Vectastain Elite ABC kit and AEC peroxidase substrate. AEC was used for the single immunohistochemical labelling in this study to maintain consistency with the double-labelled sections described below. For double labelling the red of AEC is more readily distinguished from the blue second label than DAB.

B. DOUBLE LABELLING.

Double immunohistochemical labelling was performed in order to detect co-expression of steroid receptors by endometrial leucocyte populations. The technique is described in Chapter 2. Double-labelled slides were not counterstained. Table 4.2 shows the double labelling combinations prepared.

Table 4.2. *Double labelling combinations.*

	Frozen Sections		**Paraffin Sections**		
	ER-LH2	PGR	ER-LH2	ER-6F11	PGR
CD45	Yes	yes	yes	yes	yes
CD3	Yes	yes	no	yes	yes
CD56	Yes	yes	no	yes	yes
CD14	Yes	yes	no	no	no
MT-1	No	no	yes	yes	yes

All slides were initially mounted in Supermount and, after overnight drying, with DPX mountant and coverslips.

Evaluation

All sections were analysed qualitatively for double and single labelling.

Results

As described previously (Chapter 3), large numbers of CD45 positive cells were identified throughout the endometrium (Figs. 4.1 and 4.2) and decidua with aggregates adjacent to glands and vessels. CD3 positive cells were present in all the frozen tissues, with aggregates in the stratum basalis, particularly adjacent to glands. There was a marked increase in CD56 positive cells in late secretory phase and decidual tissues in comparison with proliferative endometrium, findings complimented by the increased number of MT-1 positive cells in formalin-fixed paraffin-embedded tissues. MT-1 also reacted with T cells in endometrium. Only small numbers of CD14 positive cells were identified in all frozen tissues studied.

In frozen and formalin-fixed tissues oestrogen receptor was expressed in both epithelium and stroma in proliferative and secretory phase endometrium as previously described (Chapter 3) (Fig. 4.3). Oestrogen receptor staining was present throughout the decidual stroma and oestrogen receptors were expressed by glandular epithelial cells of decidualised endometrium (Fig. 4.4).

Progesterone receptor was variably expressed in some glands in proliferative endometrium, but glands generally showed little expression in the luteal phase as previously described (Chapter 3). Progesterone receptors were expressed throughout the stroma in both phases of the cycle (Figs.4.5 and 4.6) in non-pregnant

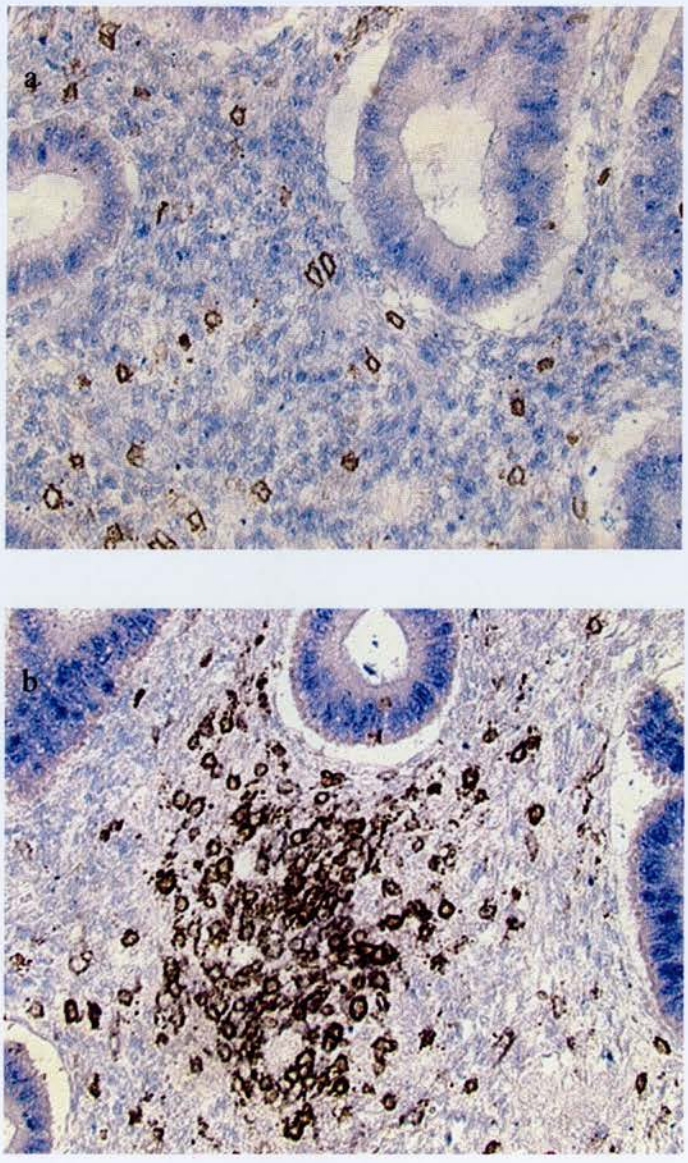


Figure 4.1. *Paraffin wax sections of secretory endometrium. Double labelled;CD45 (red), ER-6F11 (blue).* a) *(x20);* b) *(x20).*

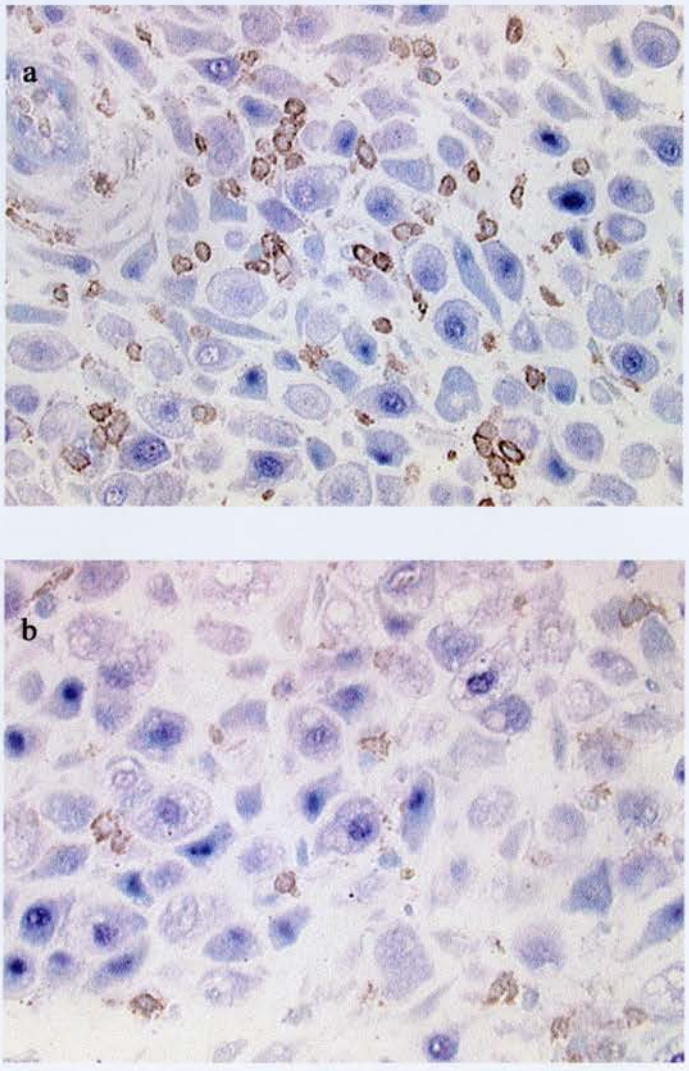


Figure 4.2. *Paraffin wax sections of early pregnancy decidua. Double labelled.* a) *PGR (blue), CD45 (red), (x40);* b) *PGR (blue), MT-1 (red), (x40).*

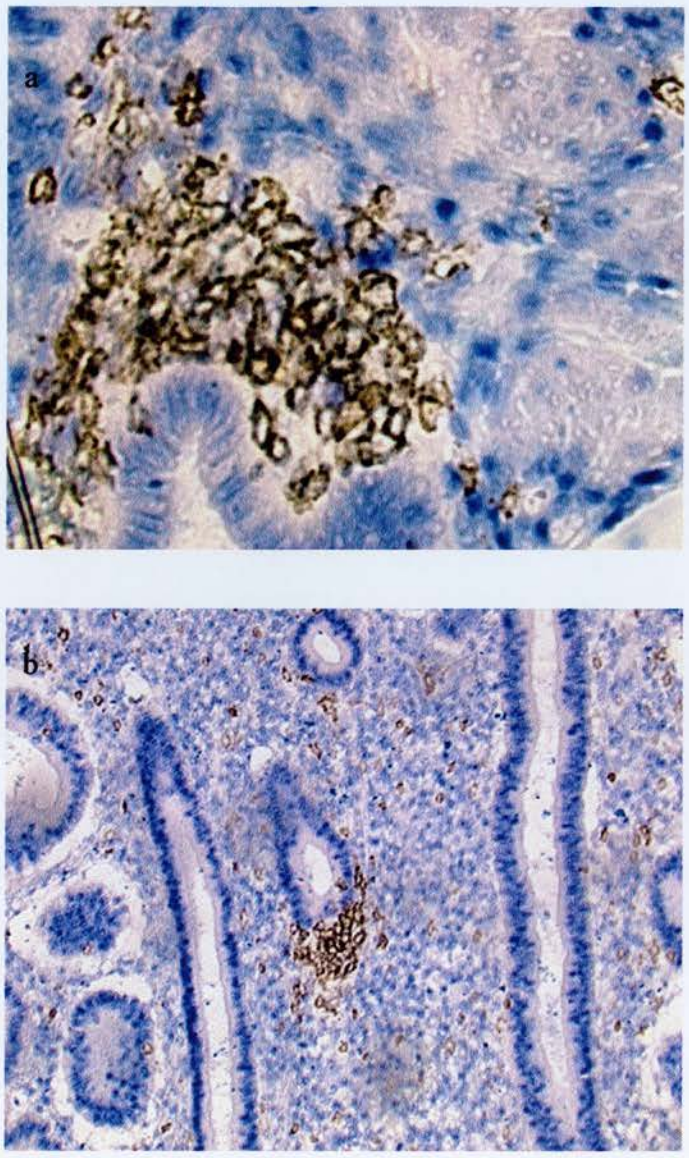


Figure 4.3. *Paraffin wax sections of endometrium. Double labelled; MT-1 (red); ER-6F11 (blue).* a) *(x40);* b) *(x10).*

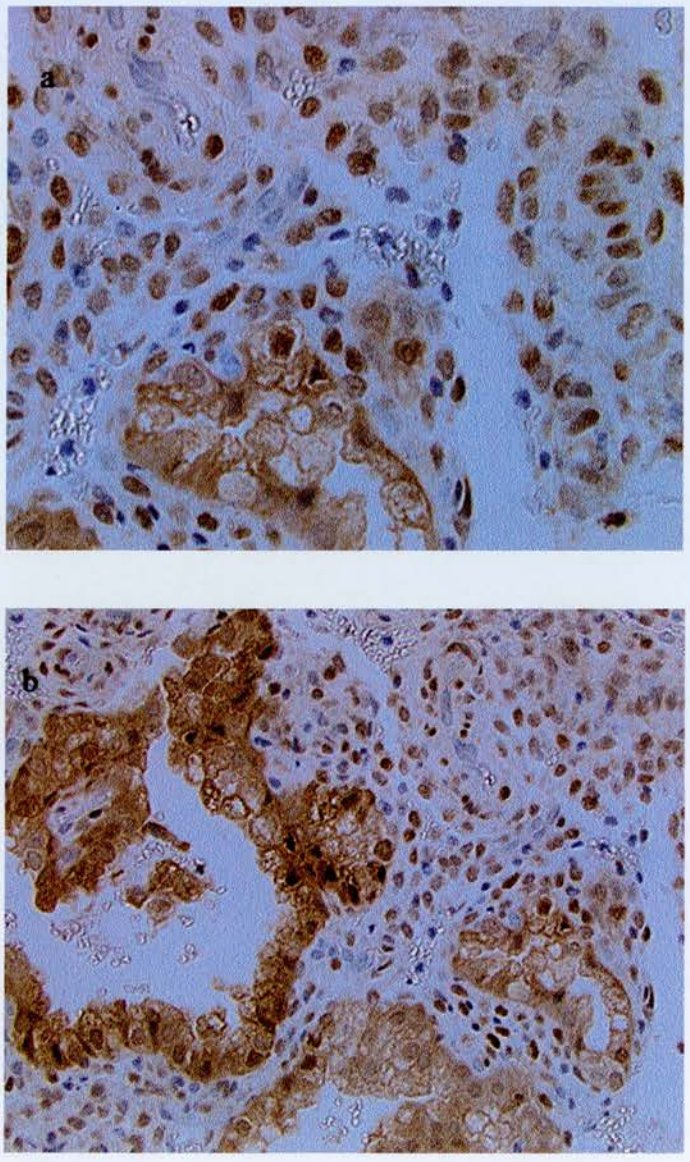


Figure 4.4.*Paraffin wax sections of decidua. Oestrogen receptor labelled brown.* a) *(x40);* b) *(x20).*

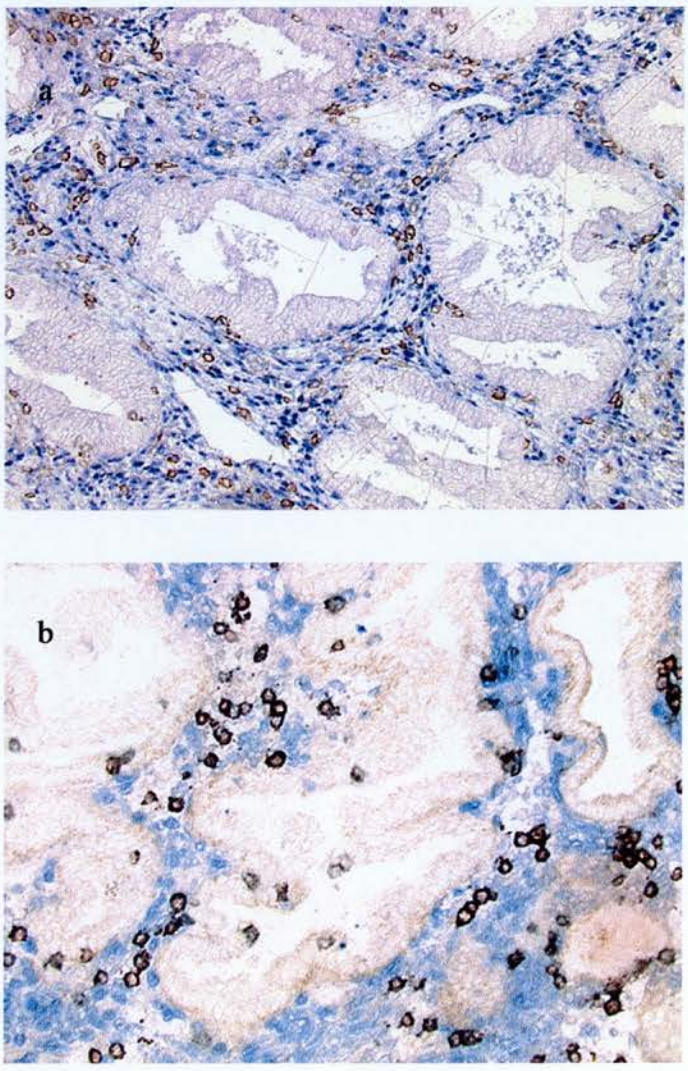


Figure 4.5. *Double labelling in secretory phase endometrium.* a) *Paraffin wax section, PGR (blue), CD45 (red) (x20);* b) *frozen section, PGR (blue), CD56 (red) (x20).*

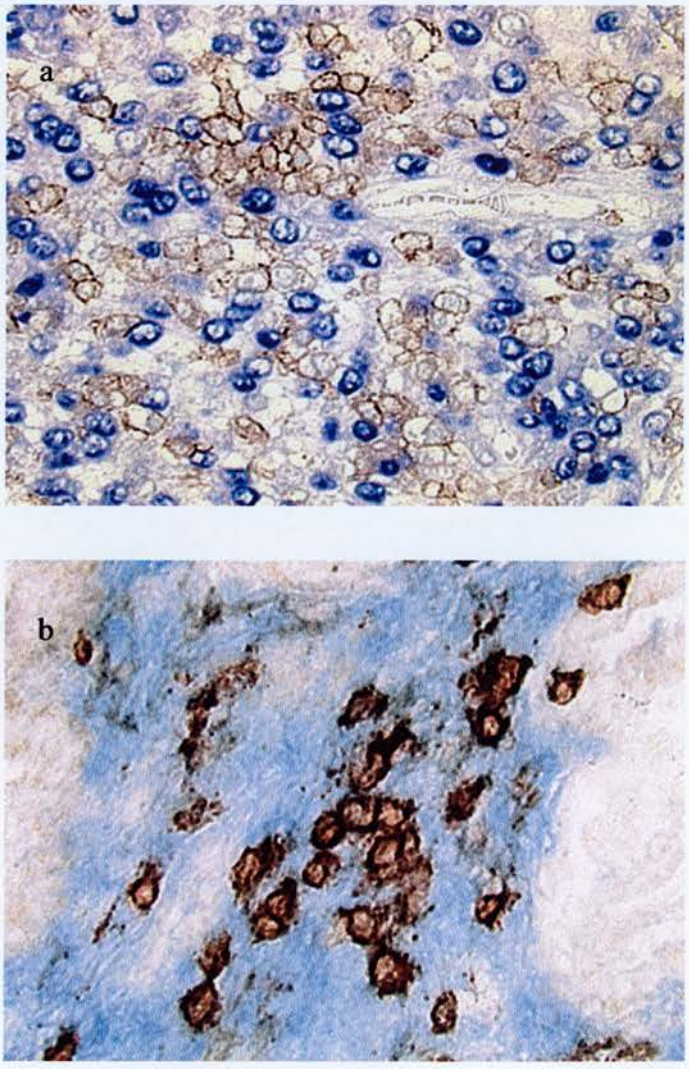


Figure 4.6. *Double labelling in secretory phase endometrium.* a) *Paraffin wax section, PGR (blue), MT-1 (red), (x40);* b) *frozen section, PGR (blue), CD45 (red), (x40).*

endometrium and throughout the decidual stroma (Fig. 4.2). These results are in keeping with those described in Chapter 3 in Pipelle endometrial samples.

The results of single labelling of frozen and paraffin-embedded sections for both leucocyte subpopulations and steroid hormone receptors were in keeping with these observations and those documented previously (Bulmer et al. 1991b, Coppens et al. 1993, Wu et al. 1993).

There was no evidence of double labelling for ER or PR by any of the leucocyte populations in any of the sections examined (Figs. 4.1, 4.3, 4.5 and 4.6). In particular, the T cell aggregates in the stratum basalis (CD3 positive and MT-1 positive) did not show steroid receptor binding (Fig. 4.3). CD56 positive cells (and MT-1 positive in paraffin sections) did not express oestrogen or progesterone receptors at any stage of the cycle (Figs. 4.3, 4.5 and 4.6) or in early pregnancy decidua (Fig. 4.2).

2. Bcl-2 Expression in Endometrial Leucocytes

Introduction

CD56 positive eGLs have been said to be undergoing apoptosis in the late secretory phase because of their unusual morphological appearance at that time, the nuclei exhibiting changes compatible with apoptosis – becoming shrunken, dark and pyknotic (King et al. 1989, Loke and King 1995). This would parallel, for example, the apoptosis occurring in lymph nodes to remove excess T and B lymphocytes following cessation of a specific immune response (Ekert and Vaux 1997). This

suggestion, however, contradicts evidence that eGLs continue to proliferate up to LH+13 (Klentzeris et al. 1992, Pace et al. 1989) and continue to function premenstrually (Jones et al.1997c).

Gompel et al. (1994) demonstrated endometrial stromal and epithelial cell expression of bcl-2 in particular noting maximal stromal expression in the secretory phase. Koh et al. (1995) attributed the rise in endometrial stromal bcl-2 expression to the proliferation of endometrial CD56 positive leucocytes, demonstrating co-localisation of bcl-2 and CD56 in secretory phase endometrial stroma by double immunohistochemical labelling. Bcl-2 is known to play a role in normal immune cell function (Reed et al. 1994, Ekert and Vaux 1997).

This study was designed to assess dual expression of CD56 and bcl-2 in endometrial stromal tissue and to clarify the argument that proliferating eGLs may also be undergoing apoptosis in the late secretory phase of the menstrual cycle. T cells and macrophages have not been examined in this study because their populations do not display the large fluctuations of numbers seen in eGLs through the menstrual cycle, macrophages increasing slightly premenstrually and T cell numbers remaining fairly stable throughout.

Experimental Design

Endometrial tissue

Frozen endometrial tissues were derived from subjects recruited into fertile groups who had been sampled as described in Chapter 2 at either LH+7 or LH+13.

Monoclonal Antibodies

Antibodies to CD56 (Novocastra, Newcastle upon Tyne, UK) (1:200) and bcl-2 (Novocastra) were used.

Double immunohistochemical labelling

This was performed as described in Chapter 2.

Sections were initially labelled for bcl-2 using the ABC method developed with AEC to produce a red reaction product. Following washing in TBS for 10 minutes and incubation with blocking serum for a further 30 minutes, the slides were incubated with the second primary antibody, namely anti-CD56. In this case the ABC alkaline phosphatase kit was used, the alkaline phosphatase substrate kit III (Vecta Blue) producing a blue reaction product. The sections were mounted with Supermount, dried and subsequently mounted with DPX and coverslips. Double labelling negative controls were performed as described in the previous section of this chapter. Tonsil sections were used as positive controls for bcl-2 labelling.

Evaluation

Sections were examined qualitatively for double labelling.

Results

Although a small proportion of CD56 positive cells also expressed the bcl-2 antigen, the majority did not at either LH+7 or LH+13. In addition there was an excess of

bcl-2 labelled cells in stroma (around 60% of the total) which were not CD56 positive (Fig.4.7). This was the case at both LH+7 and LH+13.

Discussion

The cyclical effects of the sex steroids oestrogen and progesterone on the endometrium are integral to the menstrual cycle and the histological changes have been well documented. Immunohistochemical studies have characterised the glandular and stromal distribution of sex steroid receptors during the menstrual cycle. Although the cyclical changes in endometrial leucocyte populations are dramatic and have also been well documented, the mechanisms controlling these changes have not been defined.

Studies of endometrial steroid receptor expression generally make no specific mention of the leucocytes which comprise a large proportion of the stromal cells, particularly in the late secretory phase of the cycle. Tabibzadeh and Satyaswaroop (1989) reported that a small proportion (1-7%) of T lymphocytes in basal endometrial lymphoid aggregates expressed oestrogen receptors. This contrasts with results of the present study in which sex steroid receptors were not detected on any group of endometrial leucocytes, and which showed absence of oestrogen receptors on T lymphocytes in lymphoid aggregates in the basal layer of the endometrium. The discrepancies in results between these two studies could be due to differences in the immunohistochemical techniques used. Tabibzadeh and Satyaswaroop (1989) used peroxidase-labelled secondary antibodies for both primary antibodies in double immunohistochemical labelling. In the present study two different enzyme systems

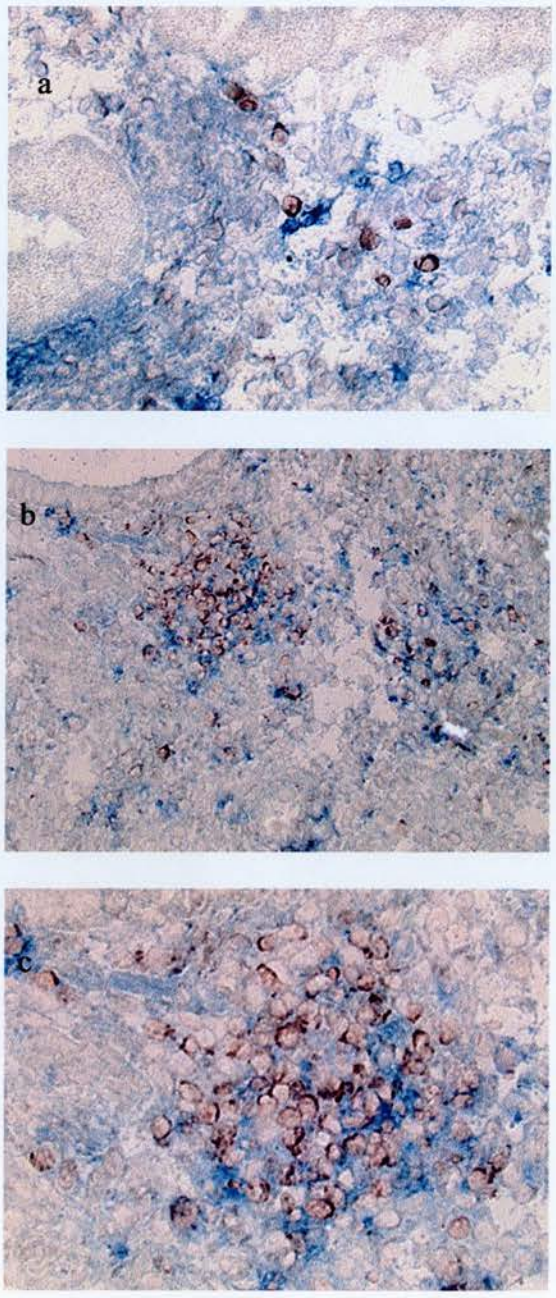


Figure 4.7. *Frozen sections of Pipelle endometrial samples. Double labelled; CD56 (blue), bcl-2 (red).* a) *LH+7 (x40);* b) *LH+13 (x20);* c) *LH+13 (x40).*

for immunolabelling were used. Furthermore, endogenous peroxidase activity was revealed by 4-chloro-1-naphthol resulting in a blue reaction product (Tabibzadeh and Satyaswaroop 1989) and it is not clear how this was differentiated from specific staining with CN-H_2O_2 which also produced a blue reaction product. The red and blue reaction products used for double immuno-staining techniques in the present study were easier to interpret than blue with the brown/black of diaminobenzidine (DAB). Differences may also result from use of different monoclonal antibodies and their specificities. The present study used two anti-oestrogen receptor antibodies (ER-LH2 and ER-6F11) and two antibodies to detect endometrial granulated lymphocytes (CD56 and MT-1). Consistent and compatible results were obtained throughout the study. The use of both paraffin and frozen sections indicate that the tissue preparation alone is not responsible for the negative findings.

King et al. (1996) reported on endometrial leucocyte steroid receptor expression with similar results. Fixed tissue was examined and double immuno-staining techniques similar to those used here were used although the study was confined to labelling for oestrogen and progesterone receptors and CD45 (leucocyte common antigen) because of the limitations of anti-CD56 in fixed sections. Furthermore, the authors failed to demonstrate specific oestrogen receptor labelling in decidual tissue. This may be due to the relative unsuitability of ER-LH2 for paraffin sections. In the current study it was found that a more recently developed antibody, ER-6F11, gave greatly improved labelling in paraffin-embedded endometrial sections and confirmed oestrogen receptor expression in decidual tissue as has been reported elsewhere (Wu et al. 1993).

The above findings with respect to bcl-2 expression are in keeping with other work done in this laboratory (Jones et al. 1998a) where CD56 positively selected cells from all stages of the menstrual cycle were examined for expression of the bcl-2 antigen. In the early secretory phase only 5% and in late secretory phase 16% of positively selected CD56 positive cells were found to express bcl-2 antigens. These findings confirm that a significant proportion of eGLs may be protected from apoptosis by bcl-2 antigen. In the same study (Jones et al. 1998a) also showed significant (~45%) expression of Ki67 in the late secretory phase by CD56 positive cells isolated from normal endometrium. In addition limited studies for apoptosis using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL) assay (Gold et al. 1994, Jones et al. 1998b) demonstrated apoptotic cells rarely in normal endometrial sections throughout the menstrual cycle.

Conclusion

Endometrial leucocyte populations do not appear to be directly controlled by cyclical ovarian steroid hormones oestrogen and progesterone. In addition a proportion of endometrial granulated lymphocytes have been shown to express bcl-2 antigen potentially protecting them from apoptotic demise in the secretory phase of the cycle. The majority however, fail to express this antigen.

CHAPTER 5

ENDOMETRIAL LEUCOCYTE POPULATIONS

ENDOMETRIAL LEUCOCYTE POPULATIONS AND SUB-POPULATIONS

Introduction

The endometrial stromal leucocyte population comprises endometrial granulated lymphocytes (eGLs), macrophages, T cells and a very few B cells (Bulmer 1991, Starkey et al. 1991). It is recognised that endometrial macrophage numbers increase slightly towards menstruation whilst T cell numbers remain relatively stable and eGLs show a marked increase in numbers in the secretory phase which is further enhanced in early pregnancy decidual tissue (Bulmer et al. 1991b, Starkey et al. 1991, Klentzeris et al. 1992). The putative roles of endometrial leucocytes have been discussed (Chapter 1). In view of the speculated roles of eGLs and T cells with regard to implantation, it is of interest to discover whether or not the leucocyte populations differ in women with otherwise unexplained subfertility compared with women with no known fertility problem.

If implantation is dependent on normal endometrial leucocyte function then it could be postulated that it may be hindered by altered numbers of specific leucocyte populations or alternatively by their abnormal function. This could be due to an inherent leucocyte abnormality or as a result of an abnormal stimulus affecting either their expansion or recruitment or their function, such as the NK like activity of eGLs. An excess of cytotoxic or suppressor T cells (CD8 positive) compared with T helper cells (CD4 positive) or their subsets and products or an excessive macrophage response in the secretory phase might similarly affect implantation. The following study was designed to assess the numbers and distribution of the different endometrial leucocyte populations in early and late secretory phase endometrium in

women with unexplained subfertility in order to compare this with the fertile population. The early secretory phase was represented in this study by samples of endometrium taken at 7 days post-ovulation during the implantation window, and the late secretory phase by sampling at 13 days post-ovulation, immediately pre-menstruation. One previous study of luteal phase endometrial biopsies from fertile and subfertile subjects timed from the pre-ovulatory LH surge, (Klentzeris et al. 1994) did not address the issue of previous pregnancy and nulliparous subfertile women were compared with parous fertile controls. In order to take into account the possible permanent immunological effect of previous pregnancy, as discussed previously (See Chapter 1), endometrium from parous subjects, both fertile and subfertile, was compared with endometrium from nulliparous women, again subfertile and, in this case, presumed fertile. Populations of eGLs, T cells, B cells and macrophages were considered.

Not only are the relative numbers of leucocytes in the endometrial stroma of interest, but functional status is also of importance. As has already been discussed, the controlling factors for endometrial leucocytes, in particular the unique eGLs, are poorly understood. The role of steroid hormones in this has been addressed in previous chapters (Chapters 3 and 4). Whilst a direct link was excluded, cyclical and population differences in the expression of endometrial steroid hormone receptors may be indirectly implicated. It is well known that leucocytes co-operate in normal immune responses. Central to these interactions are cytokines which are soluble polypeptides which act as local intercellular messengers primarily affecting immune cells. It is known that, for example, helper T (T_H) cells produce cytokines which have modifying effects on the function of cytotoxic (T_C) cells (Roitt 1994).

Similarly macrophages are activated by cytokines produced into their surroundings. Little is known about the factors that activate eGLs, but *in vitro*, IL-2 has been shown to activate them into cells capable of killing trophoblast, whereas without IL-2 activation eGLs show NK activity but cannot lyse trophoblast (King and Loke 1990, Ferry et al. 1991).

The activation status of leucocytes can be assessed in part by the expression of cell surface antigens. Known leucocyte activation antigens are CD69 (T lymphocytes, decidual NK cells), CD25 (IL-2R) (T-cells, B-cells, macrophages and some eGLs) and HLA-DR (a proportion of eGLs and T cells). It is of interest therefore to ascertain the relative expression of these activation markers in the stroma of fertile compared with subfertile endometrium. This is especially relevant given the finding that some early spontaneous miscarriages are associated with increased numbers of activated leucocytes determined by increased expression of decidual CD69, CD25 and HLA-DR (Vassiliadou et al. 1999).

Endometrial granulated lymphocytes display some natural killer cell activity *in vitro* but have a characteristic phenotype which differs from that of peripheral blood natural killer cells (Deniz et al 1994, Loke and King 1995) [Appendix 1]. It is possible, however, that this phenotypic difference represents a different stage in NK cell development. Endometrial NK cells may simply be less mature than NK cells in blood, although it has been recognised that the "mature" form is rarely seen in the endometrium or decidua. It is therefore possible that these cells may under certain circumstances, become more like their peripheral blood relatives which are known to

have a different cytotoxic response *in vitro* (Christmas et al. 1990). CD57 is an antigen expressed by NK cells but endometrial granulated lymphocytes exclusively are CD57 negative (King et al. 1991). Thus this marker can be used to distinguish between the two cell types and expression in the endometrium would indicate the change of eGLs to peripheral blood NK type cell. Any such changes in phenotype of eGLs converting them from specialised uterine NK cells into "classical" NK cells, could well have serious implications with regard to implantation and the establishment of pregnancy therefore. CD16 (FcγRIII) is present on most circulating peripheral blood NK cells but is detected on only a very small proportion of eGLs in endometrial sections and appears to confer a functional difference (Chapter 1).

T cell numbers have previously been shown to remain relatively constant throughout the menstrual cycle. Although Klentzeris et al. (1992) found a significant increase in CD8 positive T cells from LH+4 to LH+7, there was no significant change throughout the remainder of the secretory phase of the menstrual cycle. In addition they found that CD8 positive T cells outnumbered CD4 positive T cells throughout the secretory phase in a ratio of 2-3:1. Since T cell subtypes have different functions the assessment of CD8/CD4 balance in subfertile versus fertile endometrium would be of interest.

Subtypes of CD4 positive T helper cells are by virtue of their expressed cytokines capable of exerting different immune responses. They may be defined as T_H0, T_H1 or T_H2 cells as described previously (Introduction). The effects these cytokines may have on the overall function of the endometrium is unknown, but since T_H1 cells are

considered to be detrimental to pregnancy and T_H2 cells favourable by virtue of their cytokine production, it seems likely that an imbalance may affect fertility.

The distinction between those T cells expressing TCRγδ antigens in this situation may also be of interest. γδ T cells, which are both CD4 and CD8 negative, mediate a non-HLA restricted cytotoxic function involved in the removal of damaged cells (Christmas and Johnson 1996). These cells remain relatively constant in number throughout the menstrual cycle and in early pregnancy decidua (Vassiliadou and Bulmer 1996). Their role in this situation remains speculative but there is the possibility of an effect on trophoblast invasion and hence fertility. A change in their representation within the endometrium in relation to fertility status may therefore indicate altered function of the tissue.

Klentzeris et al. (1994) introduced the idea that leucocyte populations may differ in fertile compared with subfertile endometrium, suggesting a reduced CD8 positive/CD4 positive T cell ratio and lower numbers of eGLs in the subfertile.

Thus, in the following study a series of leucocyte markers were examined to assess specific leucocyte populations; total white cells (CD45, leucocyte common antigen), T cells (CD3) and T cell subpopulations (CD4, CD8, TCRαβ, TCRγδ), B cells (CD19), macrophages (CD14), eGLs and NK cells (CD56, CD16 and CD57) and leucocyte activation markers (CD69, CD25, HLA-DR). Four groups of women were considered in order to take into account any effect of previous pregnancy;

nulliparous subfertile, parous subfertile, nulliparous presumed fertile and parous fertile (Chapter 2).

Materials and methods

Endometrial samples

The subject groups described above (Chapter 2) were used; NF (n=7), NI (n=14), PF (n=10) and PI (n=10) and frozen sections from timed Pipelle endometrial samples (LH+7 and LH+13) examined. Not all subjects submitted to two samples.

Monoclonal antibodies

The specificities, dilutions and sources of all antibodies used are given in Table 5.1. All the antibodies used for the study had been evaluated for optimal use in frozen sections of endometrium in this laboratory previously. Leucocyte populations in each sample were identified by a single immunohistochemical staining procedure using the appropriate primary antibodies.

Immunohistochemical labeling procedures

Single immunohistochemical labeling was performed as described in Chapter 2 using the Vectastain Elite ABC kit. The enzyme reaction was developed using DAB as chromagen.

Controls

As previously, frozen sections of tonsil were included as positive controls for leucocyte markers in each immunostaining run. Normal negative controls where primary antibody was replaced with normal serum were used for each sample.

Table 5.1. *Monoclonal antibodies.*

Antibody	Specificity	Source	Dilution
CD45	Leucocyte common antigen	Dako	1:200
CD3	T lymphocytes	Dako	1:200
CD4	T helper cells	Silenus	1:200
CD8	T cytotoxic/ suppressor cells	Ortho	1:400
CD14	Macrophages	Dako	1:40
CD16	NK cell marker, FcγRIII	Becton Dickinson	1:100
CD19	B lymphocytes	Dako	1:100
CD56	Natural killer cells	Novocastra	1:200
CD57	NK cell marker	Novocastra	1:10
TCRαβ	T cell sub type	T Cell Diagnostics	1:10
TCRγδ	T cell sub type	T Cell Diagnostics	1:10
CD25	IL2-R, activation antigen	Dako	1:200
CD69	Activation antigen	Serotec	1:200
HLA-DR	Activation antigen	Dako	1:2000

Evaluation

For each slide, labelled and non-labelled stromal cells were counted in 2 high-power fields (x40), using a 10x10mm graticule and amounting to a total of approximately 1,000 cells per slide counted. Fields were randomly selected in areas of good quality staining with good morphology and including as much stroma as possible. The small size of some samples made the choice of areas quite limited. It is unlikely therefore that these sections would have proved suitable for automated analysis where a relatively large area of uniform tissue is required to give effective results. Since Pipelle endometrial samples are by nature relatively superficial and piecemeal it is difficult to identify clearly the distinction between the stratum functionalis and stratum basalis. In practice there is probably little stratum basalis represented in biopsies from premenopausal subjects. This is in keeping with the relative rarity of

leucocyte aggregates in the sections. For quantitation purposes aggregates were avoided since it was not possible to count cells in aggregates accurately. Where there were very scanty numbers of labelled cells, for example CD19 positive B lymphocytes, an estimated incidence of 1/500, 1/1000 or 0 were given. Numbers of labelled cells were expressed as a percentage of the total stromal cells.

Statistical analysis

The methods used for the statistical analysis of these data have been described in Chapter 2.

Results

Total stromal cell counts

There was no significant difference in the total number of stromal cells per high-power field counted i) between sections of the same tissue labelled with different monoclonal antibodies, ii) between different tissues from the four groups of subjects, and iii) between tissues at LH+7 and LH+13 (data not shown).

General Distribution of Leucocytes

In general the distribution of endometrial leucocytes was seen to be fairly uniform throughout the stroma in these endometrial samples. There were however, in keeping with previous studies and the findings described in Chapter 3, occasional aggregates of leucocytes. These comprised T cells and CD56 positive eGLs. In Pipelle samples these aggregates were infrequent presumably as a result of the

relatively superficial sampling of the endometrium compared with post-hysterectomy specimens where full thickness blocks of endometrial tissue are taken.

Cyclical changes

In all groups, the previously recognised changes in CD45 and CD56 positive cells in the endometrial stroma were confirmed (Figs. 5.1 and 5.2) reaching significance in most cases. There were also increases in the numbers of stromal cells expressing CD3 and CD14 from LH+7 to LH+13 in all subject groups (Fig.5.3), although these increases generally did not reach significance. There were small but insignificant changes in numbers of CD19 labelled cells (Fig 5.3) (Table 5.2).

Although there were quite marked differences in the numbers of endometrial stromal cells expressing CD45 and CD56 in our series, there appeared to be a wide variation in the number of stromal cells expressing these antigens between individuals particularly at LH+13 (Fig. 5.4). This may effectively make differences in the groups appear less significant.

Although in smaller numbers, paired samples (ie. LH+7 and LH+13 samples from the same subject) were available and were examined as such in order to take this variation into account (Table 5.3). These paired samples provided similar results to the unpaired groups and in particular did not produce unexpected significant results.

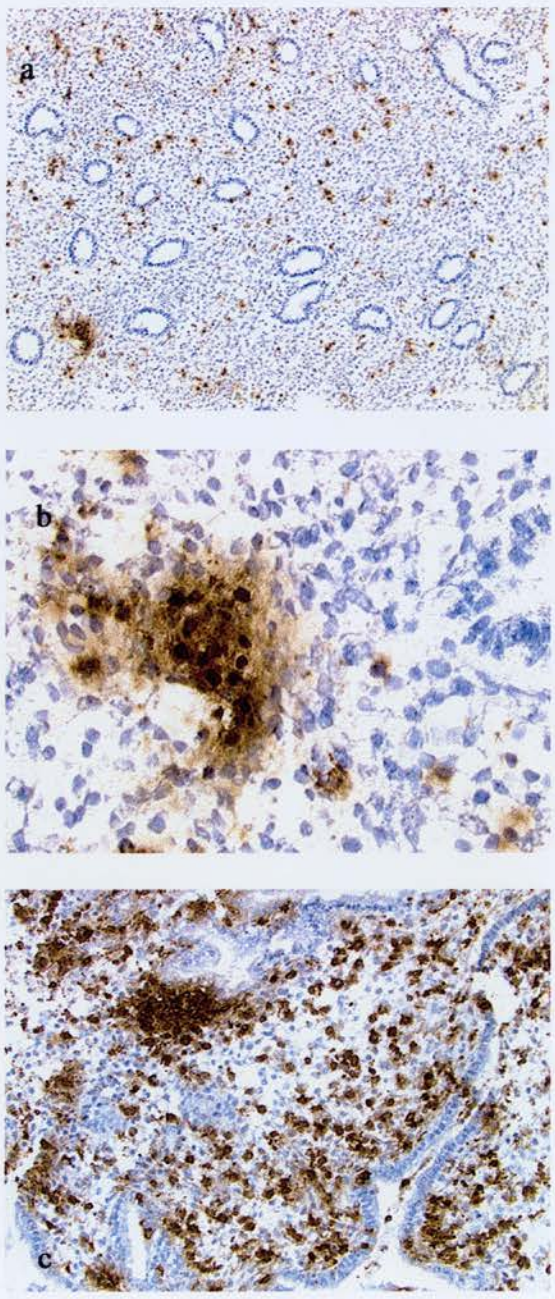


Figure 5.1. *Frozen sections of Pipelle endometrial samples, labelled for CD45.* a) *LH+7 (x10);* b) *LH+7 (x40);* c) *LH+13 (x20).*

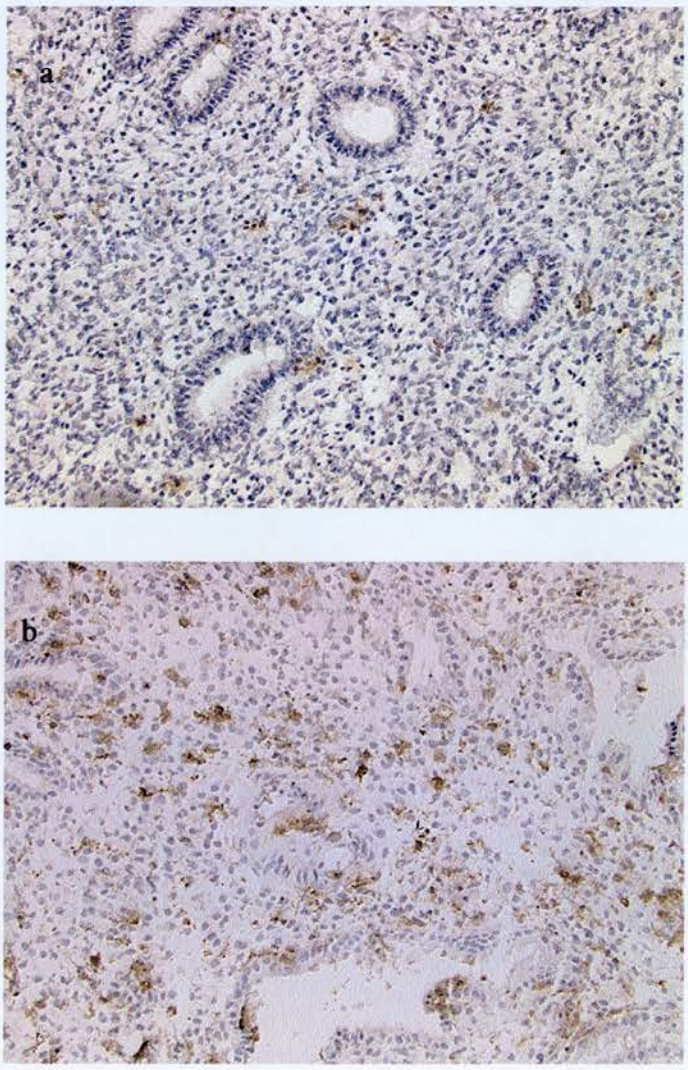


Figure 5.2. *Frozen sections of Pipelle endometrial samples. Labelled for CD56.* a) *LH+7 (x20);* b) *LH+13 (x10).*

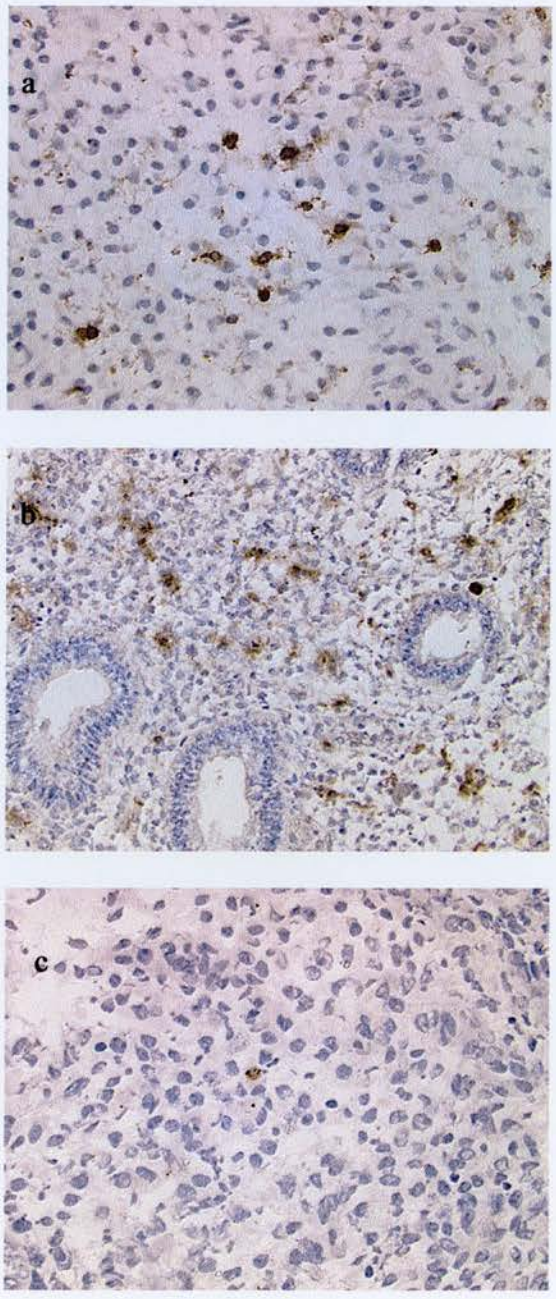


Figure 5.3. *Frozen sections of Pipelle endometrial samples. Single labelled.* a) *LH+13, CD3 (x40);* b) *LH+7, CD14 (x20);* c) *LH+7, CD19 (x40).*

Table 5.2. *Cyclical changes in endometrial leucocyte populations.*

	LH+7			**LH+13**			
	% of stromal cells labelled			% of stromal cells labelled			
	median	**mean**	**range**	**median**	**mean**	**range**	**p***
Nulliparous Fertile		n=7			n=5		
CD45	7.63	8.17	3.74-14.47	53.31	43.18	11.36-60.55	***0.010***
CD56	3.05	4.47	0.70-14.47	36.92	28.62	2.71-52.84	*0.073*
CD3	3.01	3.42	1.58-5.98	3.42	6.85	1.11-12.97	*0.164*
CD14	1.57	1.92	0.92-3.99	3.07	3.22	0.85-6.57	0.527
CD19	0.10	0.09	0-0.20	0	0.04	0-0.10	0.343
Nulliparous Infertile		n=12			n=10		
CD45	9.87	12.37	5.42-19.45	35.56	32.24	4.28-68.43	*0.107*
CD56	6.96	7.22	1.86-16.05	33.25	29.59	1.20-60.27	***0.023***
CD3	3.21	3.47	1.52-6.69	4.82	5.39	2.46-12.59	*0.140*
CD14	2.17	2.44	0.10-5.15	2.58	3.16	0.10-12.94	0.923
CD19	0.10	0.10	0-0.50	0.10	0.11	0-0.20	0.310
Parous Fertile		n=9			n=9		
CD45	9.26	9.40	4.76-13.96	34.82	35.95	4.03-56.93	***0.006***
CD56	4.55	4.39	2.16-6.49	29.76	34.69	6.29-73.13	***<0.0005***
CD3	3.24	3.38	2.10-4.49	4.99	6.34	1.28-11.47	***0.021***
CD14	2.85	2.66	0.10-5.08	3.29	3.39	0.40-20.33	0.481
CD19	0.10	0.08	0-0.20	0.10	0.10	0-0.20	0.574
Parous Infertile		n=10			n=5		
CD45	7.42	8.09	3.32-13.03	35.89	31.11	8.11-46.65	***0.013***
CD56	6.44	8.46	2.87-17.96	35.88	30.68	3.94-45.84	*0.106*
CD3	3.31	3.89	1.58-6.93	5.66	5.09	1.41-8.32	0.503
CD14	2.63	3.54	0.47-7.61	6.91	8.27	0.99-15.49	*0.199*
CD19	0.05	0.06	0-0.20	0.10	0.13	0.10-0.20	*0.142*

*Mann-Whitney. Reaches significance at $P \leq 0.05$.

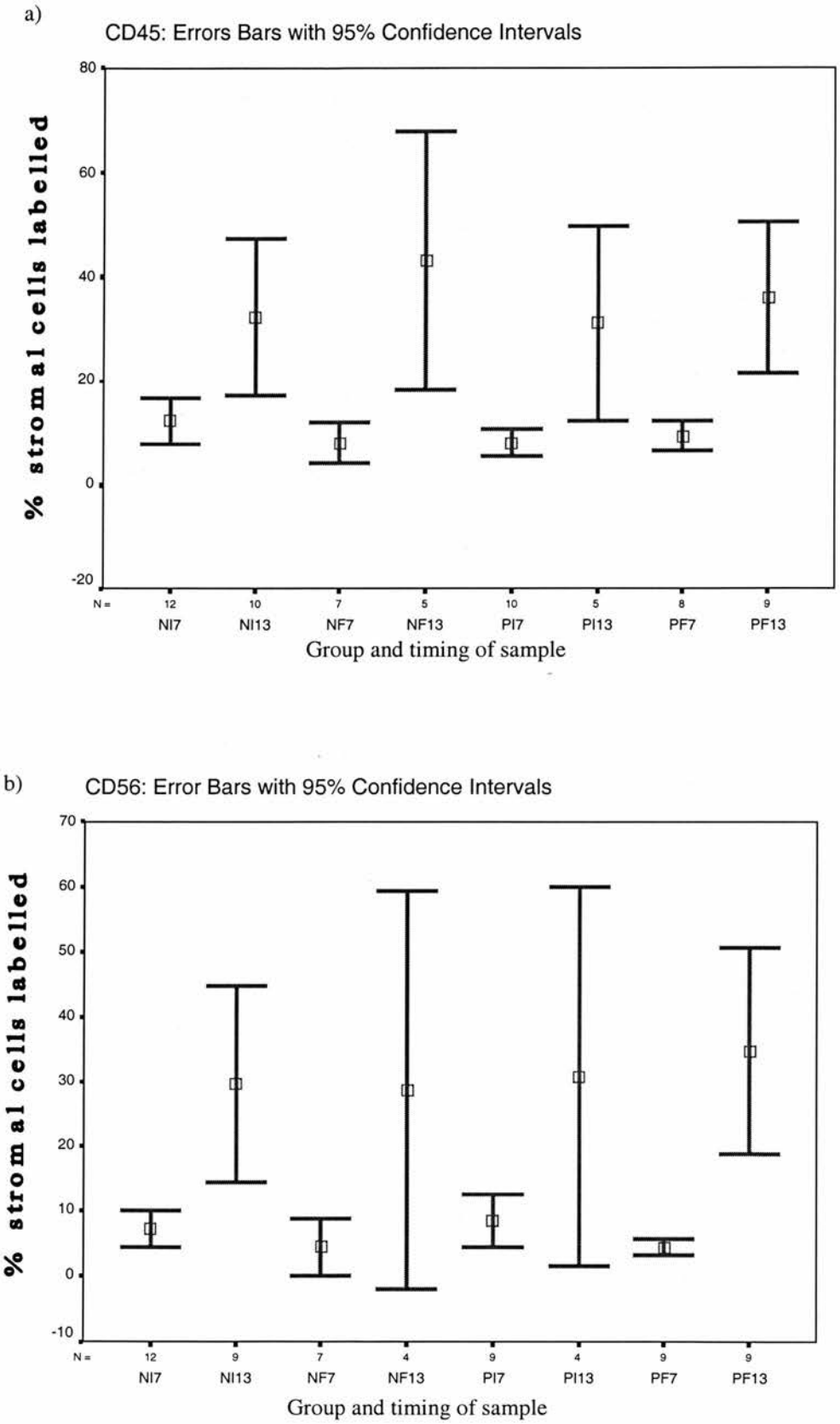


Figure 5.4. *95% confidence intervals for the numbers of a) CD45 and b) CD56 positive cells. All groups.*

Table 5.3. *Endometrial leucocyte populations. Changes from LH+7 to LH+13 in paired samples.*

		NF (n=*4;**5)		NI (n=*6;**7;***8)		PF (n=*6;**7;***8)		PI (n=*3;**4;***5)	
% of stromal cells labelled		LH+7	LH+13	LH+7	LH+13	LH+7	LH+13	LH+7	LH+13
CD45	mean	8.99	43.18**	12.22	30.40***	9.02	34.51**	7.84	31.12***
	p		***0.011***		*0.085*		***0.010***		***0.020***
CD56	mean	6.24	28.62*	6.95	30.56**	4.39	35.52***	11.33	39.59*
	p		*0.061*		***0.027***		***0.004***		***0.014***
CD3	mean	3.24	6.85*	3.79	4.71***	3.55	6.20**	3.78	5.09**
	p		*0.100*		0.426		*0.065*		0.540
CD14	mean	2.07	3.22*	2.68	3.20***	2.80	3.55**	4.02	8.27**
	p		0.471		0.750		0.465		0.243
CD19	mean	0.10	0.04**	0.13	0.01*	0.08	0.08*	0.05	0.13**
	p		0.208		0.695		1.000		*0.058*

2-tailed, paired t-test. Significance achieved where $P \leq 0.05$.

Nulliparous versus parous

There were no significant differences in the numbers of stromal cells expressing CD45, CD3, CD14, CD56 or CD19 when parous and nulliparous endometrium was compared in either fertile or subfertile subjects, at either LH+7 or LH+13.

Infertile versus fertile

There appeared to be no significant differences in the endometrial leucocyte populations between fertile and subfertile groups when parity was taken into account. What is of interest, however, was a trend for subfertile endometrium to include greater numbers of CD56 positive leucocytes at LH+7 than their fertile counterparts but to mount a smaller increase in CD56 positive leucocyte numbers by LH+13. This is reflected in similar increments in CD45 positive cells in fertile compared with subfertile endometrium (Figs. 5.5 and 5.6).

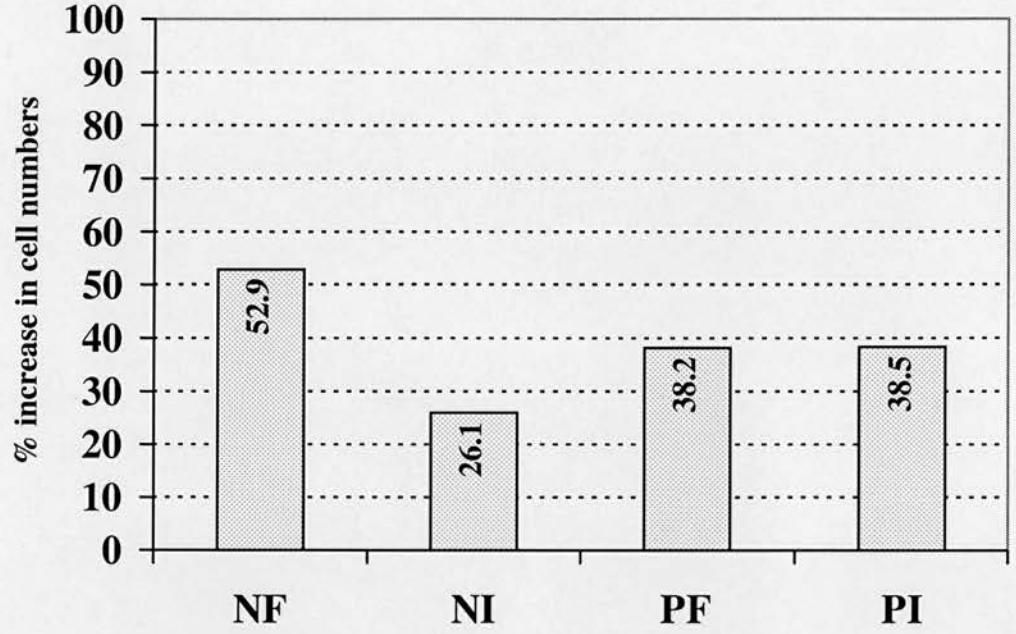


Figure 5.5. *CD45 changes from LH+7 to LH+13.*

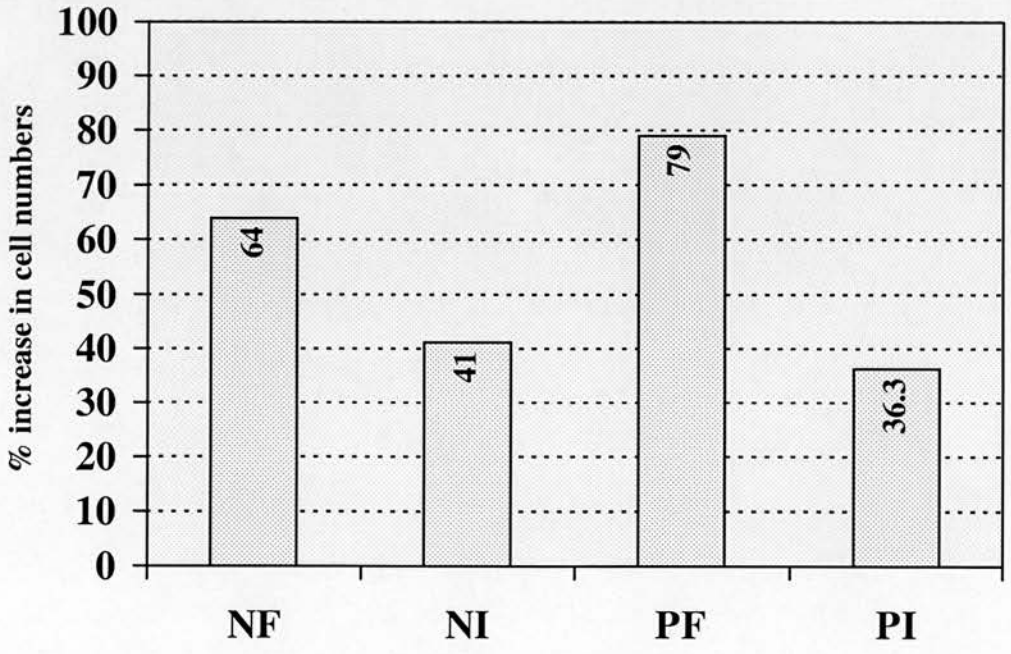


Figure 5.6. *CD56 changes from LH+7 to LH+13.*

Nulliparous infertile versus parous fertile

These findings do not confirm the results reported by Klentzeris et al. (1994), where using a different evaluation technique these authors reported significantly fewer CD56 positive cells in subfertile endometrium compared with fertile controls. The subject groups used in that study did not take into account previous parity. Although no clear difference has been shown as a result of previous pregnancy in this study, nulliparous subfertile and parous fertile groups were compared in order to provide a direct comparison with this previous study.

The trends previously described for all subfertile groups were observed when comparing parous fertile and nulliparous subfertile groups. Greater mean numbers of labelled cells were observed in the subfertile group at LH+7 and a greater increase in expression of CD45 and CD56 in the fertile group at LH+13 (Fig.5.7). These trends did not reach significance. The findings still did not confirm those reported by Klentzeris et al. (1994). There were no differences in the total numbers of stromal cells at either stage of the cycle in these groups (data not shown).

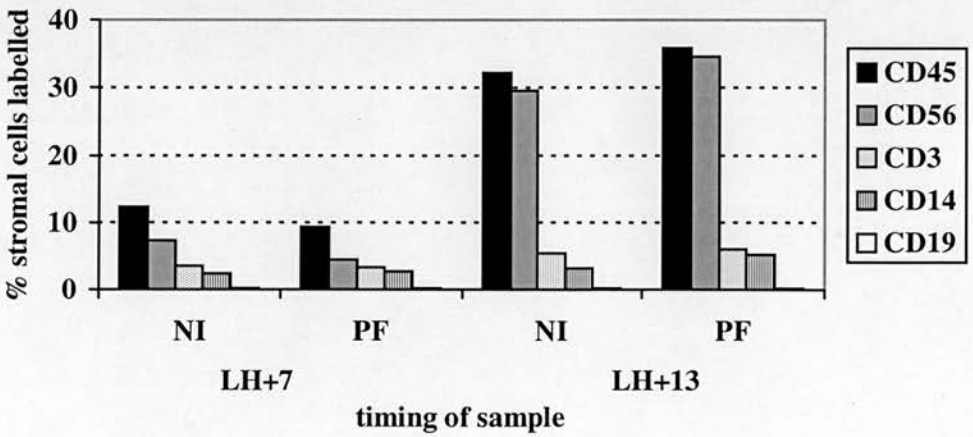


Figure 5.7. *Endometrial stromal leucocyte populations. Nulliparous infertile versus parous fertile.*

T-cell subpopulations

As described above, irrespective of previous pregnancy there was an increase in the number of CD3 positive cells in both fertile and subfertile endometrium and this increase reached significance in the parous fertile group (p=0.021) (Table 5.4). The number of helper T (T_H) cells (CD4 positive) were calculated by subtracting the number of CD8 positive (cytotoxic T [T_C] cells) from the total number of T cells (CD3 positive). The reason for this was that CD4 antibodies may also label macrophages giving a falsely high result and in the frozen tissues studied there was not a ready distinction between the two types of cells (T cells and macrophages) based on morphology alone. There were no significant differences in the numbers of T_C or T_H (Fig. 5.8) cells between the stages of the cycle examined in these groups although the increase in expression of each of these approached significance in the parous fertile group (T_H; p=0.167, T_C; p=0.167) (Fig. 5.9). This is in keeping with the work of Klentzeris et al. (1992) who showed non-significant increases in T cell subtypes over this period of the menstrual cycle. It is of interest however, that the CD8:CD4 ratio was lower at LH+13 compared with LH+7 in all groups (Fig. 5.10).

There were no significant differences in T cell sub-populations between the groups when fertility status or previous parity was considered at either LH+7 or LH+13. In addition when nulliparous subfertile and parous fertile groups were compared there were again no significant differences established at either LH+7 or LH+13.

The T cell sub-types of TCR$\alpha\beta$ or TCR$\gamma\delta$ did not appear to be cycle dependent although TCR$\gamma\delta$ positive T cells were very rare in all groups (Figs. 5.9 and 5.11).

Table 5.4. *Cyclical changes in endometrial T cell sub-populations.*

	LH+7			**LH+13**			
	% of stromal cells labelled			% of stromal cells labelled			
	median	**mean**	**range**	**median**	**mean**	**range**	**p***
Nulliparous Fertile		n=7			n=5		
CD3	3.01	3.42	1.58-5.98	3.42	5.70	1.11-12.97	*0.164*
CD4	3.86	3.44	0.29-6.15	4.40	4.82	1.68-10.00	0.537
CD8	2.19	2.16	1.37-2.88	3.75	3.61	1.04-6.32	*0.230*
TCRαβ	2.14	2.13	1.30-2.90	1.90	2.02	1.40-3.08	1.000
TCRγδ	0.10	0.10	0-0.20	0.10	0.13	0.10-0.20	0.762
Nulliparous Infertile		n=12			n=10		
CD3	3.21	3.47	1.52-6.69	4.82	5.39	2.04-12.59	*0.140*
CD4	2.29	2.33	0.42-5.61	1.49	2.11	0.48-6.11	0.536
CD8	1.44	2.43	0.68-7.39	1.89	2.77	0.65-7.00	0.808
TCRαβ	1.41	1.65	0.57-3.36	1.95	2.61	1.36-5.10	*0.069*
TCRγδ	0.10	0.12	0.10-0.20	0.10	0.11	0-0.20	0.882
Parous Fertile		n=9			n=9		
CD3	3.24	3.38	2.10-4.84	4.99	6.06	1.28-11.47	***0.021***
CD4	1.10	2.33	0.67-4.96	2.32	4.89	1.07-12.63	*0.167*
CD8	2.11	2.12	1.09-2.35	2.52	2.97	0.89-7.12	*0.167*
TCRαβ	2.25	2.48	1.27-4.80	1.65	2.71	0.63-7.17	0.730
TCRγδ	0.10	0.12	0.05-0.20	0.10	0.12	0.10-0.20	0.955
Parous Infertile		n=10			n=5		
CD3	3.31	3.75	1.58-5.48	5.66	5.30	1.42-6.14	0.503
CD4	2.33	2.59	0.20-6.18	1.91	2.65	0.61-6.67	1.000
CD8	2.48	2.54	0.95-4.62	2.88	3.25	1.55-6.30	0.513
TCRαβ	2.11	2.48	0.86-6.43	1.94	2.08	0.57-3.68	0.953
TCRγδ	0.10	0.11	0-0.20	0.10	0.10	0.10-0.10	0.839

*Mann-Whitney. Reaches significance at $P \leq 0.05$.

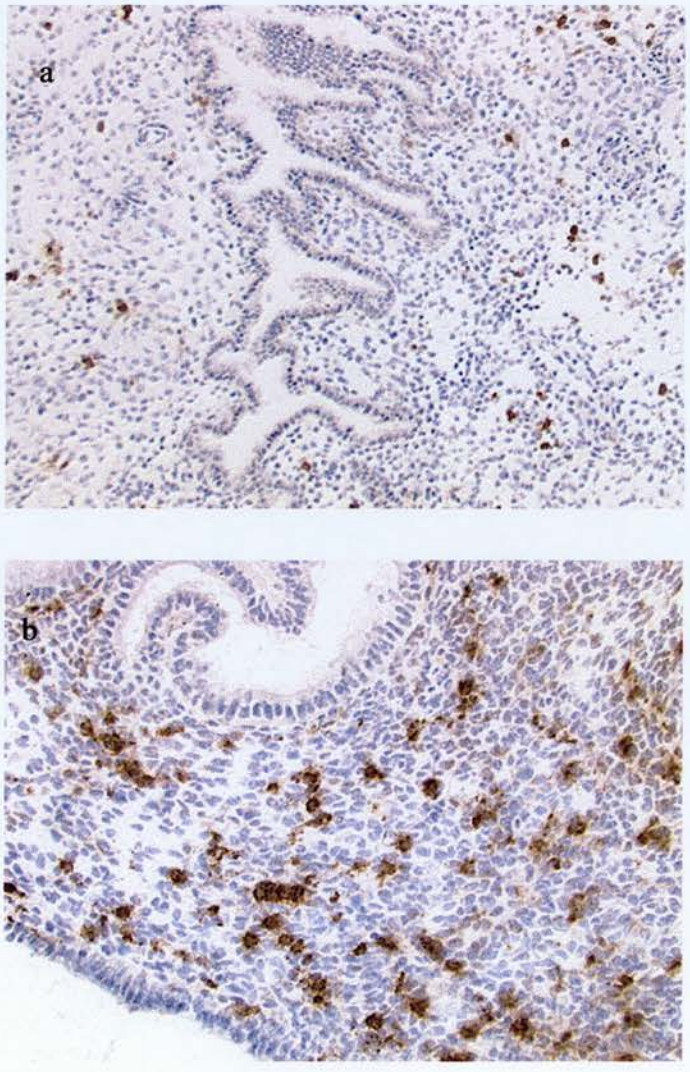


Figure 5.8. *Frozen sections of Pipelle endometrial samples. Single labelled.* a) *LH+13, CD8 (x10);* b) *LH+7, CD4 (x20).*

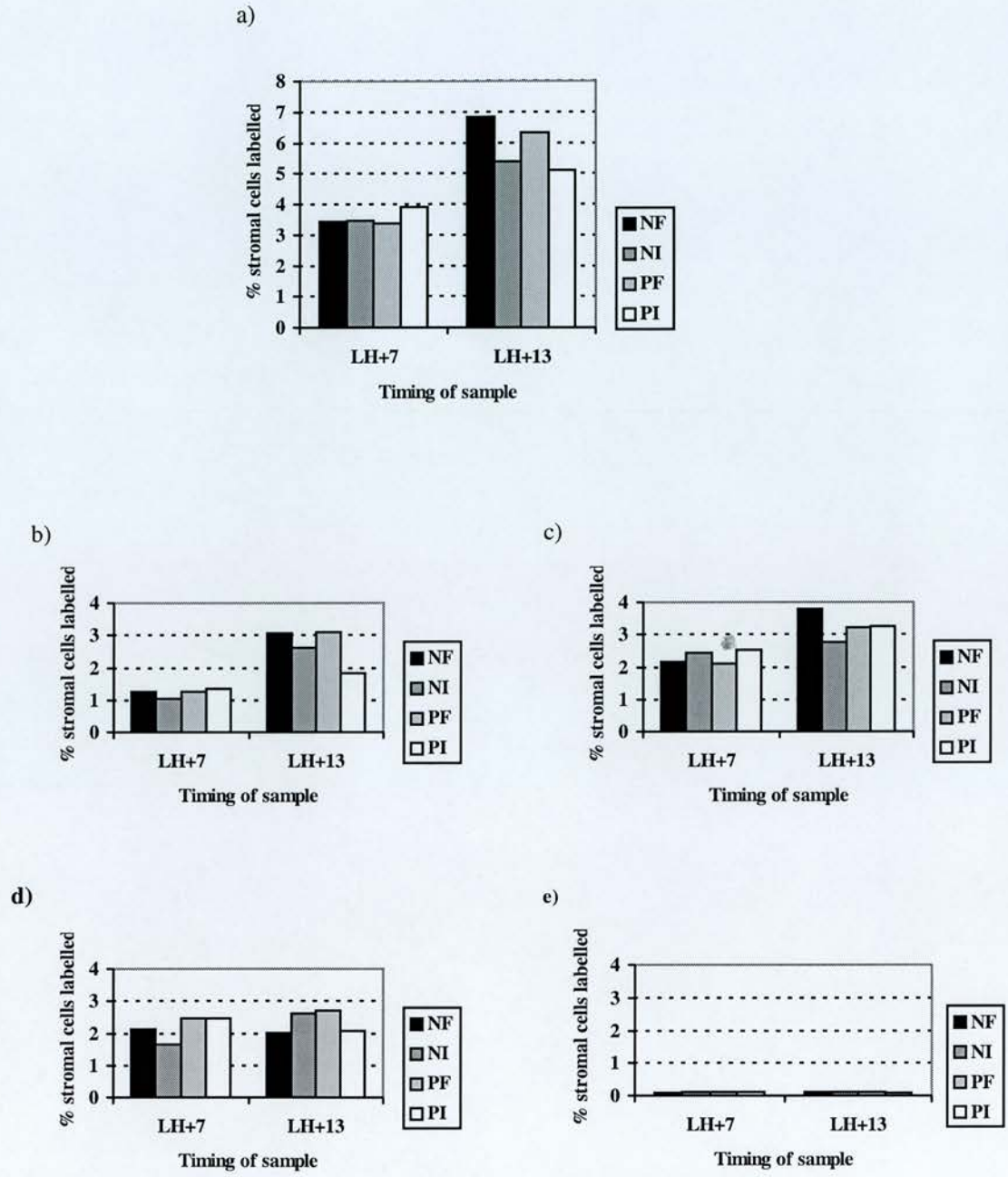


Fig. 5.9. *Endometrial stromal T cell sub-populations. Stromal labelling at LH+7 and LH+13. a) CD3, b) CD4, c) CD8, d) TCRαβ, e) TCRγδ.*

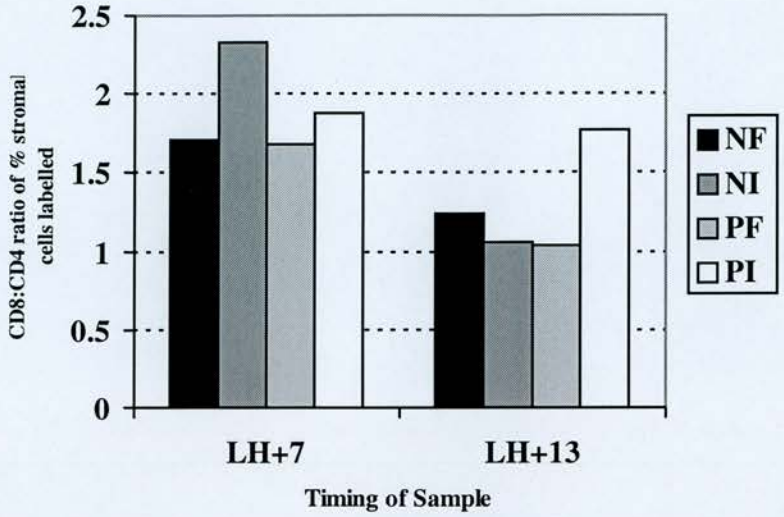


Figure 5.10. *Endometrial T cell sub-types. CD8:CD4 ratio.*

Endometrial granulated lymphocytes

CD56 labelling has already been reported (above). Anti CD16 produced unacceptable background reactivity preventing accurate assessment of its expression. Only very tiny numbers of CD57 positive cells were present in the sections studied such that their evaluation was rendered semi-quantitative, 0-1/1000 (or 2 high power fields) labelled cells being present in each section (Fig.5.12). There were no obvious differences in CD57 cell numbers between the subject groups.

Activation markers

Although only significant in the parous subfertile group (p=0.011), in each of the 4 groups of subjects the expression of HLA-DR in the stroma was seen to increase

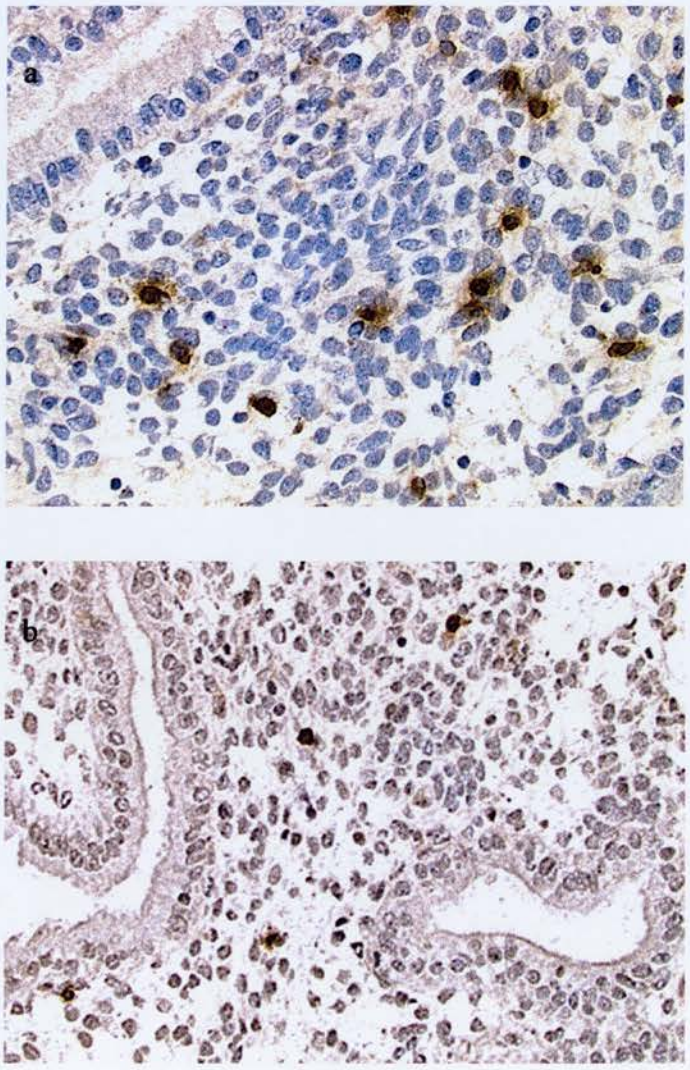


Figure 5.11. *Frozen sections of Pipelle endometrial samples. Single labelled.* a) *LH+7, TCR$\alpha\beta$ (x40);* b) *LH+7, TCR$\gamma\delta$ (x20).*

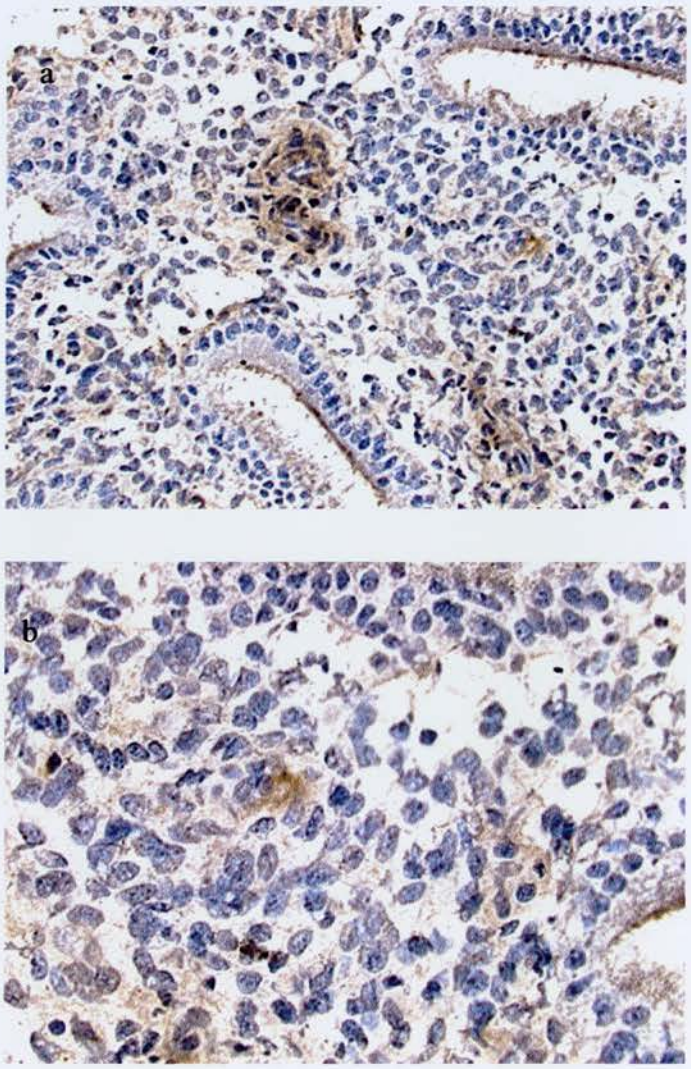


Figure 5.12. *Frozen sections of Pipelle endometrial samples, single labelled for CD57.* a) *LH+7 (x20);* b) *LH+7 (x40).*

from LH+7 to LH+13 (Figs.5.13 and 5.14). This was however, only a marginal increase in the nulliparous subfertile group (Fig.5.8). CD69 was relatively constant throughout secretory phase (Figs. 5.13 and 5.14) (Table 5.5).

In all groups CD25 expression in stroma decreased from LH+7 to LH+13 with lower expression in subfertile compared with fertile endometrium. However, only small numbers of labelled cells were present in keeping with previous accounts (Fig. 5.13) and these changes were not significant (Fig. 5.14) (Table 5.5).

Table 5.5. *Cyclical changes in endometrial leucocyte activation markers.*

	LH+7			**LH+13**			
	% of stromal cells labelled			% of stromal cells labelled			
	median	**Mean**	**range**	**median**	**mean**	**range**	**p***
Nulliparous Fertile		n=7			n=5		
HLA-DR	15.42	14.27	7.78-18.24	28.60	25.88	14.12-35.84	*0.073*
CD69	0.70	0.88	0.10-2.22	0.10	0.41	0-1.85	0.381
CD25	0.61	0.73	0-2.61	0.10	0.42	0.10-0.90	0.755
Nulliparous Infertile		n=12			n=10		
HLA-DR	13.64	13.14	3.82-24.23	15.12	13.69	0-21.42	0.503
CD69	0.13	0.44	0-1.62	0.10	0.44	0-2.67	0.600
CD25	0.20	0.38	0.10-1.11	0.10	0.20	0.10-1.00	*0.080*
Parous Fertile		n=9			n=9		
HLA-DR	11.83	17.20	5.87-28.40	17.98	22.88	14.65-37.36	*0.210*
CD69	0.69	0.72	0-2.11	0.20	0.83	0-3.30	0.622
CD25	0.37	0.74	0.10-1.87	0.10	0.36	0.10-2.14	0.321
Parous Infertile		n=10			n=5		
HLA-DR	11.49	12.11	7.63-20.49	21.58	23.78	14.77-37.22	***0.011***
CD69	0.24	0.40	0-1.04	0.10	0.38	0-1.41	1.000
CD25	0.10	0.42	0-2.65	0.20	0.33	0.10-0.92	0.513

*Mann-Whitney. Reaches significance at $P \leq 0.05$.

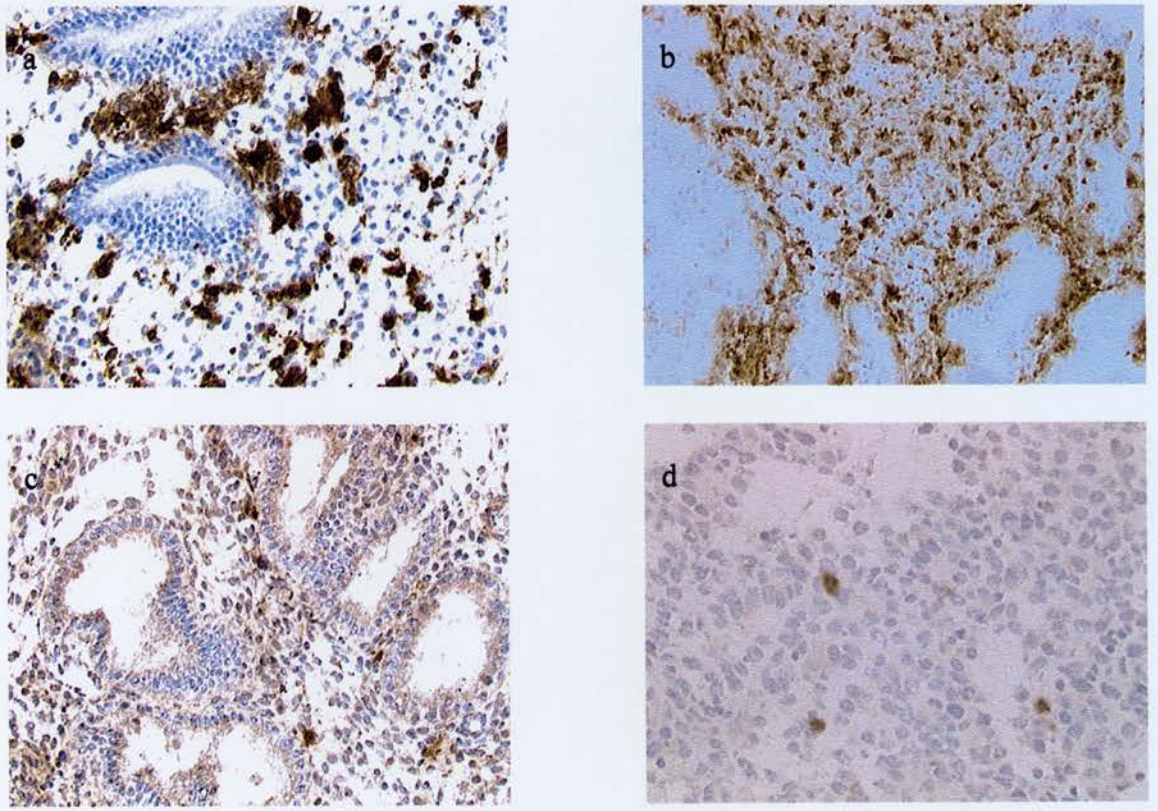
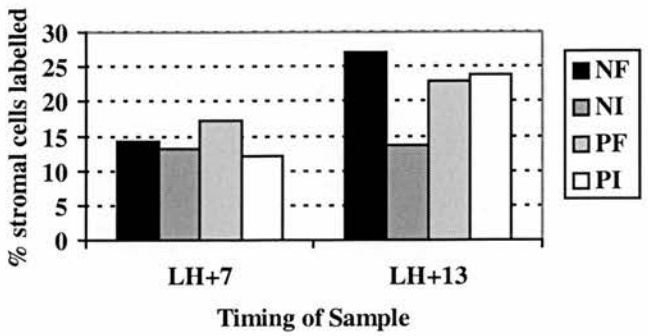
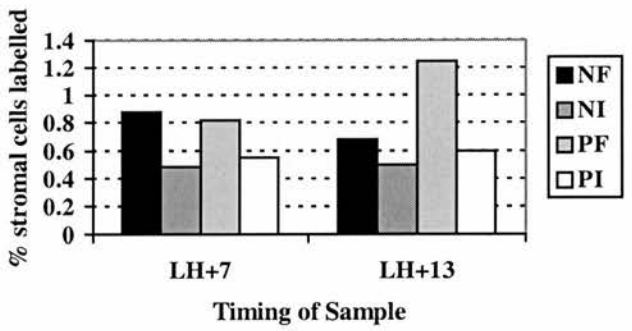


Figure 5.13. *Frozen sections of Pipelle endometrial samples. Single labelled.* a) *LH+7, HLA-DR (x20);* b) *LH+13, HLA-DR (x10);* c) *LH+7, CD69 (x20);* d) *LH+7, CD25 (x20).*

a) HLA-DR



b) CD69



c) CD25

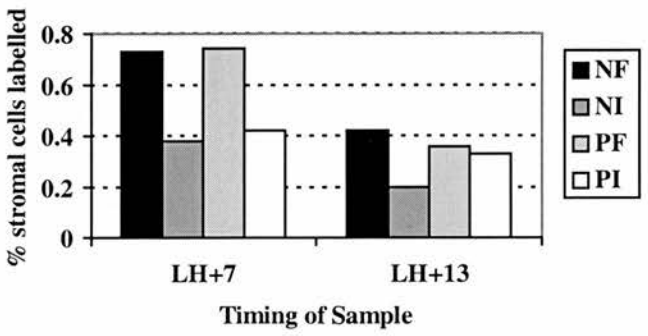


Figure 5.14. *Endometrial expression of leucocyte activation markers.*

Discussion

This study has confirmed by direct counting methods the changes previously reported in the numbers of endometrial stromal leucocytes in the luteal phase (Bulmer et al. 1991b, Starkey at al. 1991, Klentzeris et al. 1992). In addition, however, a possible increase in CD3 positive cells from LH+7 to LH+13 has also been shown, a finding which has not previously been reported. Klentzeris et al. (1992) demonstrated an increase in CD3 positive T cells and their CD8 positive, T_C, sub-type as the secretory phase of the cycle progressed. Their data show significant increases between LH+4 and LH+7, an interval not examined in the study described here. They have also reported however, a significantly lower number of CD8 positive T_C cells in subfertile compared with fertile endometrium throughout the secretory phase of the menstrual cycle (Klentzeris et al. 1994). This finding has not been confirmed even when the subject selection differences have been taken into account. The difference in the results of this study and that of Klentzeris et al. (1994) is likely to lie in the evaluation techniques. In the current study actual numbers of cells were counted rather than the use of automated techniques which are dependent on surface area of labelling and thus exposed to the vagaries of staining intensity and spread of colour beyond the cell membrane, a phenomenon which differs between antibodies and staining techniques. Furthermore cell sizes may vary between cycle stages for example as a result of proliferative activity and their relative surface area may be affected by stromal oedema. In addition in the current study results are expressed as a percentage of the total stromal cells rather than as absolute numbers of cells per field.

Despite the possibility that previous pregnancy permanently alters the immunological status of the endometrium this was not clearly reflected in leucocyte numbers. There was however, a different pattern of change in CD45 positive and CD56 positive leucocyte populations when comparing fertile and subfertile groups. Although not significant, it appeared that CD56 positive leucocyte cell numbers were more numerous around the time of implantation (LH+7) in subfertile endometrium compared with fertile but more importantly that there was a less dramatic rise in CD56 positive cell numbers as the luteal phase progressed from LH+7 to LH+13.

It is of particular interest that there was substantial variation in cell numbers expressing CD45 or CD56 between individuals at LH+13. This may be expected in the tissues studied as they were obtained in non-conception cycles. At LH+13 in such circumstances menstruation is imminent and the relevance of these cells with respect to endometrial function may be less. In addition the variation of exact timing of sampling may have a bigger impact on the observed changes at LH+13 when endometrial "breakdown" is occurring. In support of this and in contrast is the much "tighter" agreement in cell numbers at LH+7 when their contribution to endometrial function would be considered to be of greatest importance. As the actual numbers were similar in fertile and subfertile tissue the difference in expansion of the populations is likely to be a result of altered control of leucocyte proliferation. There is no direct corroborative evidence from studies of stromal Ki67 expression (Chapter 3) since, although there is a significant increase in stromal Ki67 expression from LH+7 to LH+13, fertility status does not appear to affect this.

Significant changes in T cell numbers from LH+7 to LH+13 have not been demonstrated previously and the changes in CD3 positive T cells described above were not large, numbers increasing from ~3% at LH+7 to 5% of the total stromal cell population at LH+13. These changes were only significant in the parous fertile group ($p = 0.021$) although approaching significance in the nulliparous groups (NF, NI; $p = 0.164$ and 0.140, respectively). Bulmer et al. (1991b) showed no significant change in CD3 positive cell numbers through the menstrual cycle although specimens were not timed from the LH surge but dated by Noyes criteria (Noyes et al. 1950) from the last menstrual period. Klentzeris et al. (1992) demonstrated an increase in CD3 positive cells in timed endometrial biopsies in parous fertile controls but the change, significant between LH+4 and LH+7, did not reach significance from LH+7 to LH+13. Starkey et al. (1991) showed little change in CD3 positive numbers through the menstrual cycle in frozen sections of normal endometrium examined immunohistologically. In relation to this it is of note that Klentzeris et al. (1992) identified a significant increase in CD8 positive cells though not CD4 positive cells between LH+4 and LH+7 in the secretory phase of the menstrual cycle. In that study the numbers of CD8 positive cells exceeded CD4 positive cells at all times, but the early luteal phase increase in numbers was less evident in endometrium from women with unexplained infertility (Klentzeris et al. 1994). Changes in T cell types were also seen in this study in both subfertile and fertile groups. The findings in the present study differ however, from the work of Klentzeris et al. who demonstrated a reduced CD8:CD4 cell ratio in subfertile compared with fertile endometrium. In contrast the T_C:T_H ratio demonstrated in the current study was greater at LH+7 in

subfertile compared with fertile endometrium, a finding that was not maintained at LH+13.

Most of the endometrial T cells were of αβ sub type. There are very few γδ T cells present, comprising around 0.1% of stromal cells. This is in keeping with previous studies (Chen et al. 1995, Vassiliadou and Bulmer 1996). The activation status of the T cells does not appear to be altered either by fertility status or previous parity.

The full significance these combined results will not be clear until the full functional roles of all of the cells involved are known. CD56 positive eGLs have long been believed to play a role in implantation and it may be that higher numbers of the cells at LH+7 in subfertile endometrium adversely affect this process. Lower numbers at LH+13 however, may suggest a difference in activation or function in the subfertile endometrium compared with the fertile. CD56 positive cells are NK like in activity and have been shown to proliferate in endometrium during the late luteal phase (Pace et al.1989). It has been hypothesised that their activity, in conjunction with other immune cells and regulators, may control trophoblast invasion (Liu et al. 1994). Hence the possibility that alteration in the numbers of CD56 positive cells and their functions may affect implantation and the progressive establishment of early pregnancy. The data presented show no evidence of a change in status of eGLs to a more "classical" or mature NK phenotype in relation to fertility.

Although there is no clear link between sex steroids and CD56 positive eGLs (Chapter 4) there remains the possibility that the difference in population expansion

of eGLs demonstrated in this study may be indirectly linked to, and the result of an abnormal intermediate response to those hormones. Alternatively this finding may be the result of abnormalities in the eGLs themselves and a study of Ki67 expression on eGLs in each of the subject groups would be of interest in relation to this.

It has been proposed that in mice T_H1 and T_H 2 helper T cells have contradictory roles in successful pregnancy (Wegmann et al. 1993). Thus it may not be surprising that T cell populations and ratios of T_H/T_C cells differed in subfertile compared with fertile populations. T cells produce numerous soluble factors in their role as mediators of the immune response. There are factors to which eGLs are known to be sensitive, for example IL-2 (Ritson and Bulmer 1989, King and Loke 1990) albeit present in only small amounts in the endometrium. There is also evidence for effects of T cell cytokines on placental cells (Athanassakis et al. 1987). Thus it is possible that this previously unrecognised T cell response to luteal phase, and the variation between subfertile and fertile T cell sub-populations may be responsible for altered eGL function and thus pregnancy failure via the production of a different balance of cytokines.

Any evidence for an increase in activation status of endometrial leucocytes lies in the higher HLA-DR expression at LH+13 in fertile endometrium, but this may simply parallel the increase in leucocyte numbers. No difference was seen in the early activation markers CD25 or CD69.

Conclusion

There is tight control of endometrial granulated leucocyte numbers around the time of implantation. A significant increase in eGL numbers occurs from LH+7 to LH+13 but the degree to which this expansion in the population occurs is less in subfertile compared with fertile endometrium. There is a previously unrecognised late secretory rise in T cell numbers, in both fertile and subfertile women and the balance of CD4/CD8 T cell subtypes alters between LH+7 and LH+13. Leucocyte activation status may differ with respect to HLA-DR expression at LH+13 in subfertile endometrium although the increased expression seen may be a result of cell population expansion.

CHAPTER 6

ADHESION MOLECULES IN THE ENDOMETRIUM

ADHESION MOLECULES IN THE ENDOMETRIUM

Introduction

Some of the potential controls of endometrial function have already been considered in previous chapters. It is presumed that central control is maintained by the ovarian cycle with its production of steroid hormones, mainly oestrogen and progesterone. This would constitute the "macro" control, but fine-tuning may be obtained by local messengers within the endometrium. Two categories of messenger can be considered; i) adhesion molecules - cell-cell or cell-matrix adhesion molecules involved in inter-cellular communication, and ii) cytokines - soluble inter-cellular transmitters of messages. Both of these could be influenced by local hormones.

Previous authors have examined the distribution of a variety of adhesion molecules with regard to their potential role in the menstrual cycle and fertility. The overall findings appear to suggest a cycle dependent distribution of the integrins $\alpha_1\beta_1$ (VLA-1, CD49a), $\alpha_v\beta_3$ (CD51/CD61) and $\alpha_4\beta_1$ (VLA-4, CD49d). Lessey et al. (1994) used these observations to define the "implantation window" and suggested that aberrant expression of these adhesion molecules occurred in some cases of infertility. Closer examination of regional expression of these integrins within the endometrium has revealed that surface epithelium is the major site for cyclical changes (Lessey et al. 1996a). This is of particular significance when considering the earliest of embryo/endometrial interactions. Klentzeris et al. (1993) have suggested that there is an absence of $\alpha_4\beta_1$ expression in subfertile compared with fertile endometrium. More recently Murray et al. (1999) have shown that $\alpha_6\beta_4$ remains constant throughout the menstrual cycle and thus probably has no role in endometrial receptivity.

The importance of such adhesion molecules within the endometrium and their cyclical changes may be in their contribution to normal immune cell function as discussed above and thus their potential effects on endometrial receptivity. eGLs express a wide array of adhesion molecules (Appendix 1). Furthermore there is a potential role for adhesion molecules in the embryo/endometrial interaction either directly – embryo derived fibronectin may act as a ligand for endometrial or even eGL $\alpha_4\beta_1$; or indirectly – ligand binding resulting in altered endometrial cell function.

Although Grosskinsky et al. (1996) have shown that the *in vitro* endometrial stromal expression of some key adhesion molecule subunits (α_1, α_3, α_5, α_6 and $\alpha_v\beta_3$) is unaffected by the direct application of steroid hormones, it is likely that their cyclical expression is linked to the hormone cycle at least indirectly. Indeed Grosskinsky also noted the induction of endometrial stromal expression of $\alpha_1\beta_1$ by some cytokines *in vitro*, namely epidermal growth factor (EGF) transforming growth factors-α and -β_1 (TGF-α and TGF-β_1) and of $\alpha_v\beta_3$ by IL-1α, IL-1β and TNF-α whilst in other *in vitro* studies (Simón et al. 1998) have demonstrated an IL-1 mediated up-regulation of β3 integrin in cultured endometrial epithelial cells.

Integrins have been the most studied of the adhesion molecules with regard to endometrial function, but if the roles of the different groups of adhesion molecules are considered it can be seen that there is scope for their involvement in the process of endometrial cyclicity if not directly in embryo adhesion and implantation. Cadherins have been considered for their potential role in embryo/endometrial

attachment and are implicated, by their role in intercellular binding, in the permissive changes in endometrial epithelium for blastocyst penetration. ICAM, VCAM and the selectins are implicated by virtue of their roles in immunoregulation (see below).

Thus in this study, in addition to the integrins discussed above, a selection of adhesion molecules have been studied, which are involved primarily in the immune response, in particular leucocyte trafficking. These may be of relevance to the behaviour of the endometrial immune system, which via local effectors may have a role to play in the expression of integrins throughout the menstrual cycle.

Fibronectin is a ligand for several integrins (including $\alpha_4\beta_1$) and has been strongly implicated in the process of trophoblast invasion (Damsky et al. 1994). Its deficiency in mice and that of its receptor leads to early embryonic loss (Hynes 1996). Aplin et al. (1988) described the distribution of fibronectin in endometrial stroma throughout the menstrual cycle. They noted its constant presence throughout the stroma in proliferative, early and late secretory phases. It was detected in blood vessels but not endometrial glands. Bilalis et al. (1996) found that this protein was absent in the endometrium of subfertile women throughout the secretory phase of the endometrium. Once again the studies of subfertile women compared a parous fertile group with a group of subfertile women of unspecified parity.

The study described in this chapter was designed to consider the distribution of adhesion molecules in the endometrium and to compare the distribution between

fertile and subfertile subjects. The effect of previous parity, as in other chapters, was also taken into account - a factor not considered in previous reports.

Experimental Design

Endometrial samples

Frozen sections from samples obtained from women in the four subject groups described in Chapter 2 were used.

Monoclonal antibodies

The antibodies, their specificities, source and concentrations used are described in Table 6.1. The specific adhesion molecules examined are detailed below.

Table 6.1. *Monoclonal antibodies.*

Antibody	**Specificity**	**Source**	**Concentration**
CD49a	$\alpha_1\beta_1$, VLA-1	Immunotec	1/100
CD49d	$\alpha_4\beta_1$, VLA-4	Serotec, Oxford, UK	1/400
CD61	$(\alpha_v)\beta_3$	Novocastra	1/100
ICAM-1	CD54	Novocastra	1/60
VCAM	CD106	Novocastra	1/100
E-selectin	CD62e	Novocastra	1/100
P-selectin		Serotec	1/400
Fibronectin		Novocastra	1/400

A. INTEGRINS

The integrins are initially classified according to their β subunit (Hynes 1987). Those possessing the β_1 subunit are the very late activation (VLA) family which comprises the largest group. The VLA family of molecules is expressed by lymphocytes several days after activation *in vitro* (Hynes 1987).

-CD49a ($\alpha_1\beta_1$, VLA-1). This molecule binds laminin. It is a marker of T cell activation (Tabibzadeh 1990, 1991).

-CD49d ($\alpha_4\beta_1$, VLA-4). This integrin is a fibronectin receptor which also binds the vascular cell adhesion molecule-1 (VCAM-1, discussed below) at a different site. This integrin is involved in lymphocyte-endothelial cell adhesion but may also be responsible for lymphocyte aggregation (Bednarczyk and McIntyre 1990).

-CD61 (β_3). CD61 is the β subunit of the vitronectin receptor $\alpha_v\beta_3$ (CD61/CD51) which is also capable of binding fibrinogen, von Willebrand's factor and thrombospondin. It is present on most mesenchymal cells and in particular on large vessel endothelium.

B. IMMUNOGLOBULIN SUPERFAMILY;

-CD54 (ICAM-1). Intercellular adhesion molecule-1 is widely expressed on endothelial and epithelial cells. Its expression is induced by inflammation and it promotes leucocyte adhesion by binding lymphocyte function-related antigen-1 (LFA-1, $\alpha_L\beta_2$ integrin).

-CD106 (VCAM). This molecule is expressed on endothelial cells which have been activated by the cytokines interleukin-1 (IL-1) or tumour necrosis factor α (TNFα). It promotes leucocyte adhesion, binding VLA-4 (above). Deficiency of this adhesion molecule in gene knockout mice causes placental defects and is lethal to the embryo (Hynes 1996)

C. SELECTINS;

-CD62e (E-selectin, ELAM-1). These molecules are involved in the inflammatory response: E-selectin is produced by activated endothelial cells and promotes leucocyte attachment.

-CD62 (P-selectin, GMP-140, PADGEM). P-selectin is expressed by endothelial cells, platelets, monocytes and neutrophils in response to activation. It again promotes leucocyte-endothelial adhesion but also platelet, neutrophil and monocyte interactions.

Method

Single labelling immunohistochemistry was performed as described in Chapter 2. DAB was used as the substrate for the peroxidase labelling technique giving a brown reaction product. Sections were lightly counterstained with Mayer's haematoxylin, dehydrated, cleared and mounted in DPX with coverslips.

Evaluation

All the sections were examined semi-quantitatively. Expression of antigen in glandular and surface epithelium, stroma and vascular tissue was scored from 0 to 5 (Chapter 2). The distribution of staining was assessed separately for each tissue area – stroma, glandular epithelium and surface epithelium. The staining of vessels within the stroma was noted separately.

Statistical analysis

The methods employed for the statistical analysis of data obtained in this study are discussed in Chapter 2 (Materials and Methods).

Results

CD49a (VLA-1, $\alpha_1\beta_1$)

Clear cyclical changes in the expression of CD49α were demonstrated. For both fertile and subfertile endometrium there was a trend to increased stromal VLA-1 expression and reduced glandular epithelial expression from LH+7 to LH+13 (Table 6.2) (Figs. 6.1 and 6.2). It is of interest that these changes only reached significance in the subfertile groups (Table 6.3). There was strong (mean staining score 3.63-4.00) labelling of vessels for CD49α at both LH+7 and LH+13.

When comparing fertile and subfertile endometrium there was a trend to lower expression of CD49α throughout the tissue in subfertile endometrium at LH+7 (not significant) but there was no difference in expression between groups at LH+13 (Table 6.2). The most striking feature in these data is that, although scanty at most, there was no surface epithelial expression of CD49α at LH+13 (Figs. 6.1 and 6.2). Parity did not appear to affect the expression of this antigen in endometrium (Table 6.2).

Table 6.2. *Endometrial CD49a expression. LH+7 and LH+13.*

		LH+7			LH+13		
		Stroma	**Glandular epithelium**	**Surface epithelium**	**Stroma**	**Glandular epithelium**	**Surface epithelium**
NF	N	4	4	4	2	2	2
	Mean	1.88	3.88	0.25	2.75	2.25	0
	Median	1.75	4.00	0.25	2.75	2.25	0
	Range	1-3	3.5-4	0-0.5	2.5-3	1.5-3	0-0
NI	N	8	8	8	8	8	8
	Mean	1.38	3.06	0.38	2.31	2.69	0
	Median	1.00	3.50	0	2.75	2.75	0
	Range	1-2	0-4	0-1.5	1-3	0.5-4	0-0
PF	N	6	6	6	6	6	6
	Mean	1.67	3.42	1.17	2.25	2.67	0
	Median	1.50	4.00	1.00	2.50	2.50	0
	Range	1-3	2-4	0-3	1-3.5	1.5-4	0-0
PI	N	8	8	8	3	3	3
	Mean	1.38	3.50	0.13	3.33	2.67	0
	Median	1.50	3.75	0	3.00	3.00	0
	Range	0-2	2-4	0-1	3-4	2-3	0-0

Table 6.3. *CD49a expression in endometrium. LH+7 versus LH+13.*

	Stroma	**Glandular Epithelium**	**Surface Epithelium**
Nulliparous fertile			
P	0.267	*0.133*	0.533
Nulliparous infertile			
P	***0.038***	0.574	0.234
Parous fertile			
P	0.394	0.240	*0.180*
Parous infertile			
P	***0.012***	*0.085*	0.776

Mann-Whitney. Results reach significance when $P \leq 0.05$.

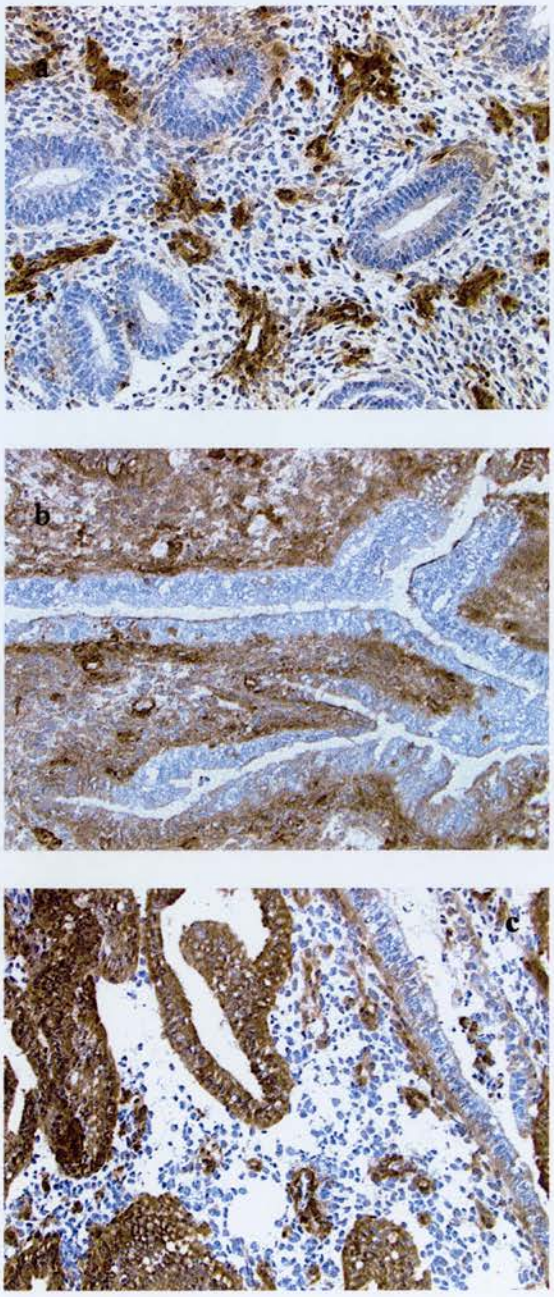
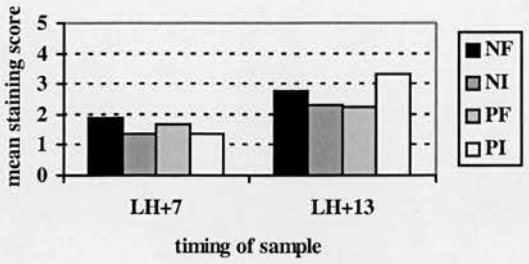
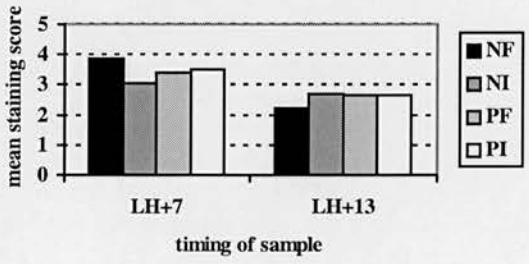


Figure 6.1. *Frozen sections of Pipelle endometrial samples. Single labelled for CD49a (VLA-1, $\alpha_1\beta_1$)* a) *Infertile, LH+7 (x20);* b) *fertile, LH+13 (x20);* c) *infertile, LH+13 (x20).*

a) stroma



b) glandular epithelium



c) surface epithelium

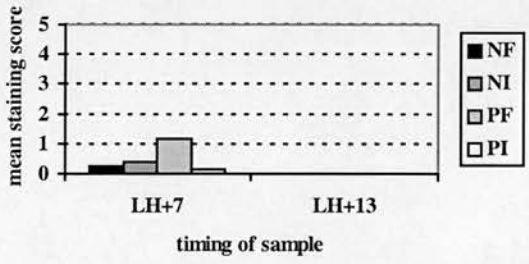


Figure 6.2. *Endometrial CD49α expression at LH+7 and LH+13. a) stroma, b) glandular epithelium and c) surface epithelium.*

CD49d (VLA-4, $\alpha_4\beta_1$)

VLA-4 expression again showed a cyclical pattern (Table 6.4) (Figs. 6.3 and 6.4). There was a trend to increased stromal and reduced epithelial expression at LH+13 compared with LH+7. The differences were not significant in most cases (Table 6.5), however, and there were also no significant differences between the four groups of subjects. There was again relatively strong labelling of vessels for this antigen (mean staining score 3.00-4.00) at both LH+7 and LH+13.

Table 6.4. *Endometrial VLA-4 expression. LH+7 and LH+13.*

		LH+7			**LH+13**		
		Stroma	**Glandular epithelium**	**Surface epithelium**	**Stroma**	**Glandular epithelium**	**Surface epithelium**
NF	N	4	4	3	4	4	4
	Mean	2.25	2.50	0.83	2.00	1.38	0.50
	Median	2.00	3.00	1.00	2.00	1.00	0.50
	Range	2-3	0-4	0-1.5	1.5-2.5	0.5-3	0-1
NI	N	10	10	9	6	6	5
	Mean	1.80	3.00	0.78	2.17	1.25	0
	Median	2.00	2.75	0	2.25	1.25	0
	Range	1-2	2.5-4	0-2.5	1.5-2.5	0-2	0-0
PF	N	6	6	4	8	8	8
	Mean	2.17	2.00	0.88	2.38	0.88	0.13
	Median	2.00	2.50	0.25	2.50	1.00	0
	Range	1.5-3	0-3	0-3	1.5-2.5	0-1.5	0-0
PI	N	9	9	7	4	4	3
	Mean	2.22	3.78	1.36	2.63	2.00	0.67
	Median	2.00	4.00	1.50	2.50	1.50	0
	Range	1.5-3	3-4	0-3	2.5-3	1-4	0-2

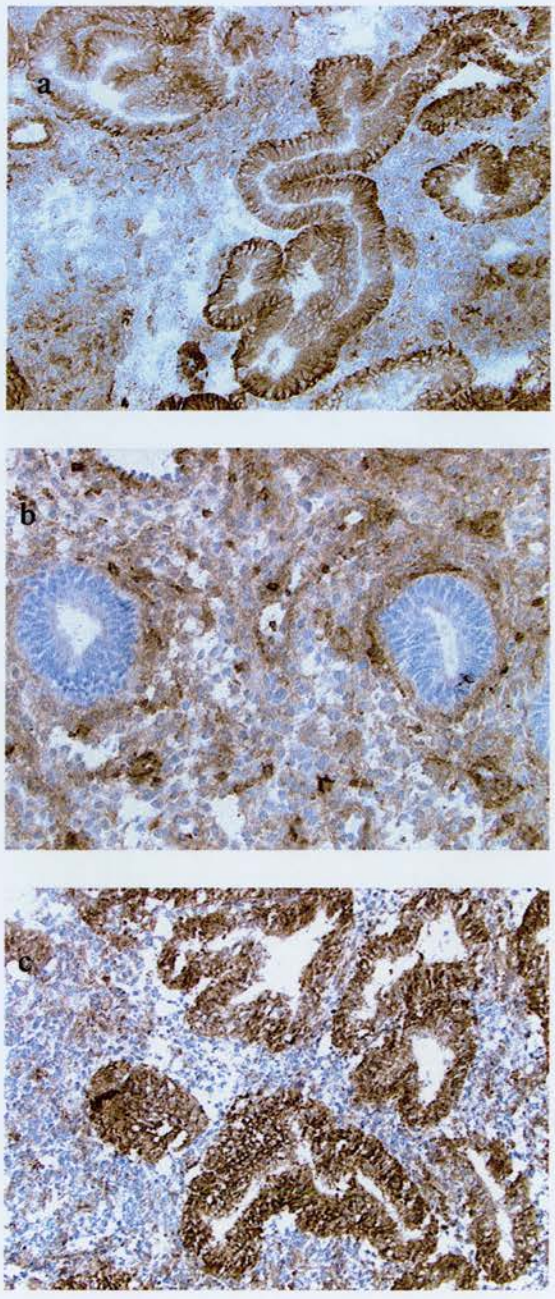
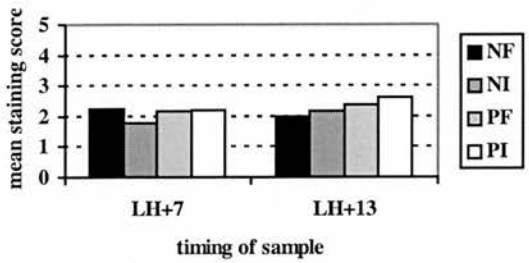
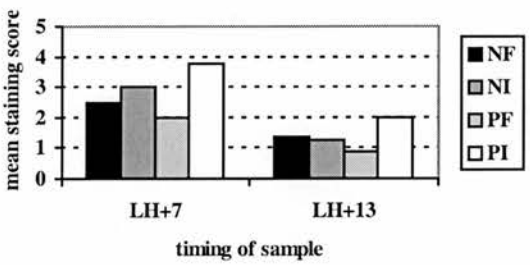


Figure 6.3. *Frozen sections of Pipelle endometrial samples. Single labelled for CD49d (VLA-4 , $\alpha_4\beta_1$).* a) *Infertile, LH+7 (x20);* b) *fertile, LH+7 (x20);*

a) stroma



b) glandular epithelium



c) surface epithelium

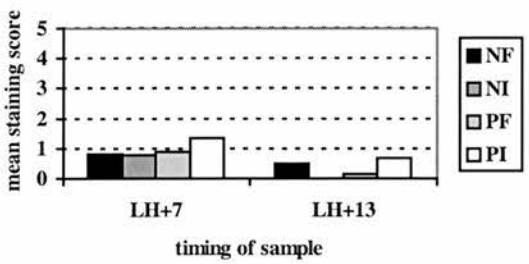


Figure 6.4. *Endometrial expression of VLA-4 at LH+7 and LH+13. a) stroma, b) glandular epithelium and c) surface epithelium.*

Table 6.5. *Endometrial VLA4 expression. LH+7 versus LH+13.*

	Stroma	Glandular epithelium	Surface epithelium
Nulliparous fertile			
P	0.686	0.486	0.629
Parous fertile			
P	*0.345*	*0.108*	*0.368*
Nulliparous infertile			
P	*0.118*	***0.003***	*0.190*
Parous infertile			
P	*0.148*	*0.076*	*0.383*

Mann-Whitney. Results reach significance when $P \leq 0.05$.

CD61 (β_3 subunit of $\alpha_V\beta_3$)

There were significant cyclical changes in the expression of CD61 in endometrium (Table 6.6) (Figs. 6.5 and 6.6). Although not quite reaching significance in the parous subfertile group, there were otherwise significant increases in the expression of CD61 in both glandular and surface epithelium from LH+7 to LH+13 (Table 6.7).

Table 6.6. *Endometrial CD61 expression. LH+7 and LH+13.*

		LH+7			LH+13		
		Stroma	**Glandular epithelium**	**Surface epithelium**	**Stroma**	**Glandular epithelium**	**Surface epithelium**
NF	N	6	6	6	5	5	5
	Mean	1.42	0.25	0.25	1.00	2.20	2.20
	Median	1.25	0	0	1.00	3.00	3.00
	Range	1-2	0-1.5	0-1.5	1-1	0-3	0-3
NI	N	11	11	11	7	7	7
	Mean	1.09	0.68	0.41	1.21	2.07	1.86
	Median	1.00	0	0	1.00	2.50	2.00
	Range	1-2	0-2	0-2	1-2	0.5-3	0-3
PF	N	8	8	7	9	9	7
	Mean	1.38	0.44	0	1.39	1.67	1.57
	Median	1.00	0	0	1.00	1.50	1.00
	Range	1-3	0-2.5	0-0	0-2.5	0-3	0-3
PI	N	8	8	7	4	4	3
	Mean	1.06	0.31	0.21	1.00	1.50	2.00
	Median	1.00	0	0	1.00	1.50	3.00
	Range	1-1.5	0-2	0-1	1-1	0-3	0-3

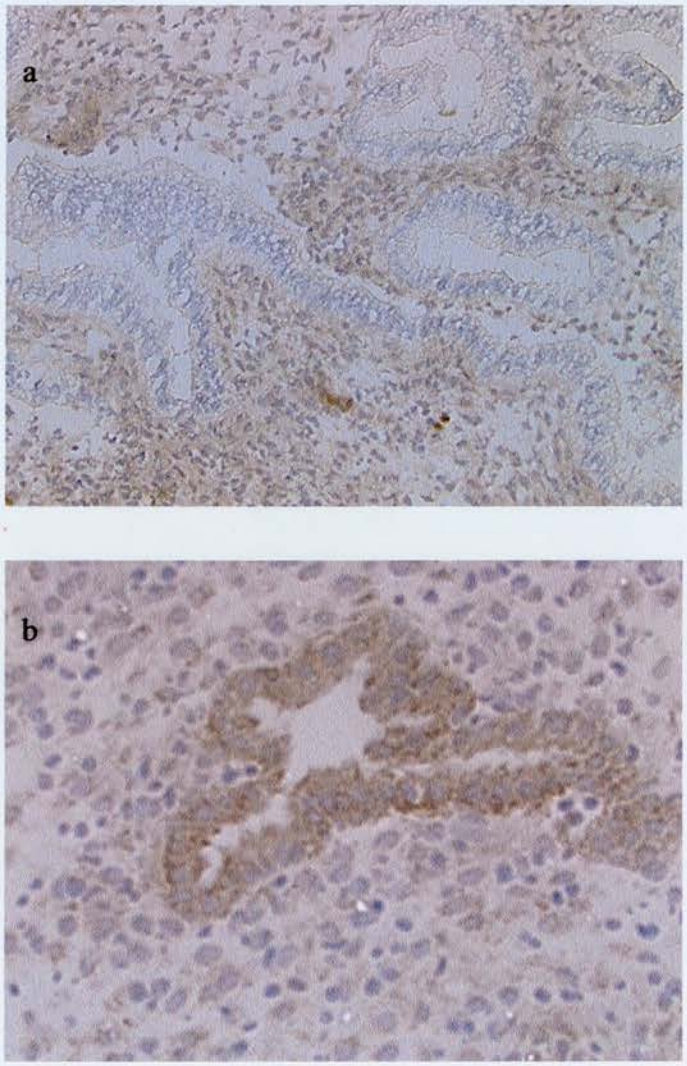
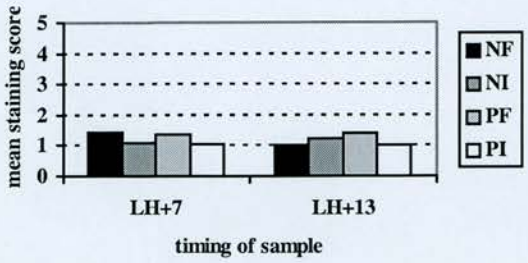
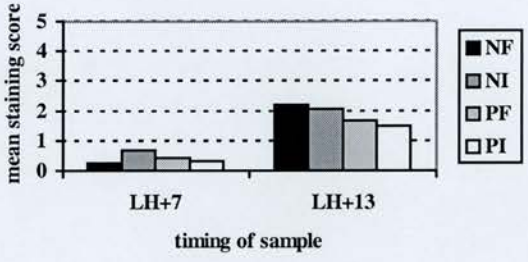


Figure 6.5. *Frozen sections of Pipelle endometrial samples. Single labelled for CD61.* a) *LH+7 (x20);* b) *LH+13 (x40).*

a) stroma



b) glandular epithelium



c) surface epithelium

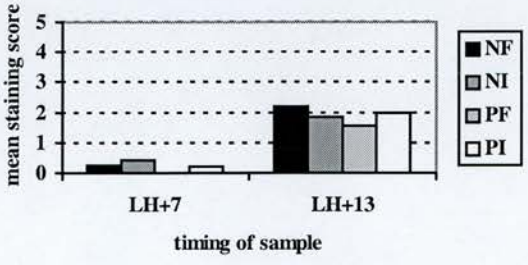


Figure 6.6. *Endometrial expression of CD61 at LH+7 and LH+13. a) stroma b) glandular epithelium and c) surface epithelium.*

Table 6.7. CD61 expression in endometrium. LH+7 versus LH+13.

	Stroma	Glandular epithelium	Surface epithelium
Nulliparous fertile			
P	*0.177*	***0.030***	***0.030***
Parous fertile			
P	0.815	***0.015***	***0.026***
Nulliparous infertile			
P	0.536	***0.008***	***0.011***
Parous infertile			
P	0.808	*0.109*	*0.183*

Mann-Whitney. Results reach significance when $P \leq 0.05$.

There were no significant differences between nulliparous and parous endometrium or between fertile and subfertile endometrium. Vessels were labelled uniformly at LH+7 and LH+13.

ICAM and VCAM

The greatest ICAM expression in the endometrium was in the vascular endothelium (mean staining score 3.63-4.00) elsewhere being of a low level (Fig. 6.7). When the population subgroups were considered there appeared to be no cyclical change in ICAM expression (Table 6.8) and the only significant change shown was in nulliparous subfertile endometrial stroma between LH+7 and LH+13 (p=0.043).

VCAM expression also appeared to have no particular cyclical pattern again being of a relatively low level but including in vascular tissue (Fig. 6.7, Table 6.9) and neither parity nor fertility appeared to exert an effect on this.

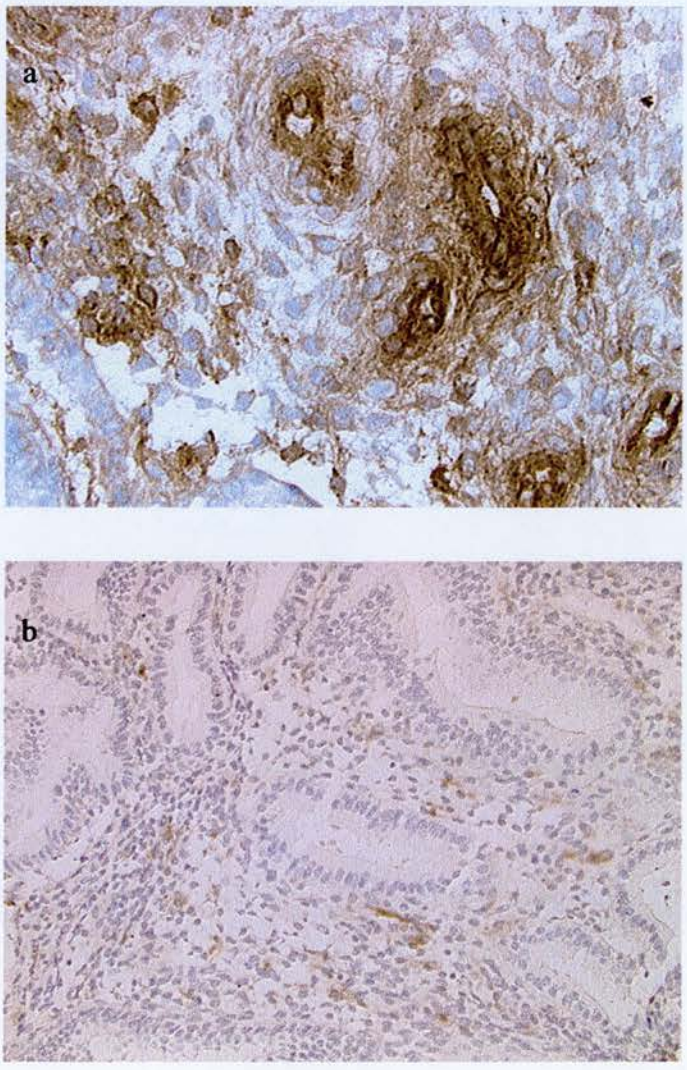


Figure 6.7. *Frozen sections of Pipelle endometrial samples. Single labelled.* a) *LH+13, ICAM (x40);* b) *LH+7, VCAM (x20).*

Table 6.8. *Endometrial ICAM expression. LH+7 and LH+13.*

		LH+7			LH+13		
		Stroma	**Glandular epithelium**	**Surface epithelium**	**Stroma**	**Glandular epithelium**	**Surface epithelium**
NF	N	6	6	5	5	5	4
	Mean	1.58	0.17	0.10	1.60	0.40	0.13
	Median	1.75	0	0	2.00	0	0
	Range	1-2	0-1	0-0.5	1-2	0-1	0.0.5
NI	N	10	10	10	7	7	6
	Mean	1.90	0.15	0	1.14	0.50	0.17
	Median	2.00	0	0	1.00	0	0
	Range	1-3	0-1.5	0-0	1-2	0-1.5	0-1
PF	N	7	7	7	9	9	7
	Mean	1.86	0	0	1.61	0.28	0
	Median	1.50	0	0	1.50	0	0
	Range	1-4	0-0	0-0	1-3	0-1.5	0-0
PI	N	7	7	7	3	3	3
	Mean	1.93	0.29	0	1.67	0	0
	Median	2.00	0	0	2.00	0	0
	Range	1-3	0-2	0-0	1-2	0-0	0-0

Table 6.9. *Endometrial VCAM expression. LH+7 and LH+13.*

		LH+7			LH+13		
		Stroma	**Glandular epithelium**	**Surface epithelium**	**Stroma**	**Glandular epithelium**	**Surface epithelium**
NF	N	6	6	5	5	3	3
	Mean	1.08	0.25	0.20	1.10	0.83	0.33
	Median	1.00	0	0	1.00	0	0
	Range	1-1.5	0-1.5	0-1	1-1.5	0-2.5	0-1
NI	N	11	8	7	7	6	6
	Mean	1.09	0.63	0.57	1.00	1.00	0.42
	Median	1.00	0	0	1.00	1.25	0.25
	Range	1-2	0-2	0-2	1-1	0-2	0-1
PF	N	8	7	7	9	7	7
	Mean	1.06	0.14	0	1.11	0.93	0.14
	Median	1.00	0	0	1.00	1.00	0
	Range	0.5-1.5	0-0.5	0-0	1-1.5	0-2	0-1
PI	N	9	8	6	4	2	2
	Mean	1.11	0.19	0	1.13	0	0
	Median	1.00	0	0	1.00	0	0
	Range	1-2	0-1.5	0-0	1-1.5	0-0	0-0

P-selectin and E-selectin

Both P- and E-selectins were expressed at low levels throughout the endometrial stroma and epithelium. Vascular labelling of P-selectin was stronger (mean staining score 3) in all groups and at both stages of the cycle examined. There were again no clear changes in expression of these adhesion molecules throughout the luteal phase (Tables 6.10 and 6.11) or between the different populations studied (Fig. 6.8).

Table 6.10. *Endometrial P-selectin expression. LH+7 and LH+13.*

		LH+7			**LH+13**		
		Stroma	**Glandular epithelium**	**Surface epithelium**	**Stroma**	**Glandular epithelium**	**Surface epithelium**
NF	n	4	4	4	4	4	2
	Mean	0.75	0.88	0.50	0.88	0.88	0.75
	Median	1.00	0.75	0.50	1.00	1.00	0.75
	range	0-1	0-2	0-1	0.5-1	0-1.5	0.5-1
NI	n	10	10	10	6	6	6
	Mean	0.65	0.70	0.45	0.75	1.50	1.00
	Median	1.00	1.00	0	1.00	1.75	1.00
	range	0-1	0-1.5	0-1.5	0-1.5	0-2	0-2
PF	n	7	7	5	9	9	7
	Mean	0.43	1.14	0.60	0.67	0.83	0.71
	Median	0	1.50	0	1.00	1.00	1.00
	range	0-1	0-2	0-2	0-1	0-2	0-2
PI	n	9	9	9	4	4	2
	Mean	1.11	0.94	0.67	1.00	0.50	0.50
	Median	1.00	1.00	1.00	1.00	0.50	0.50
	range	1-2	0-2	0-2	1-1	0-1.5	0-1

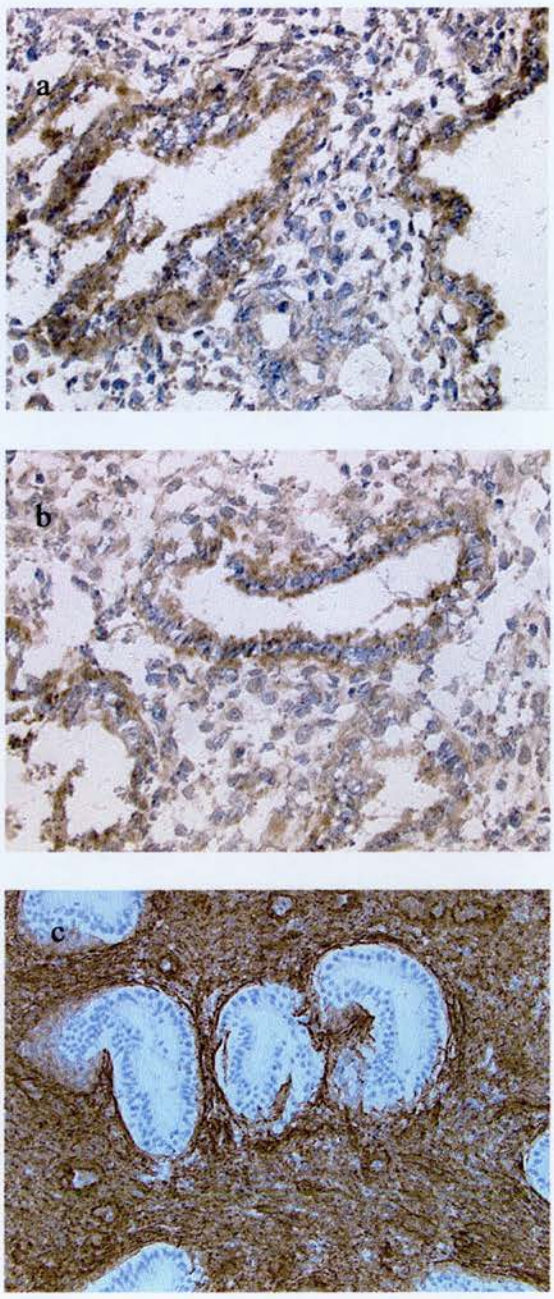


Figure 6.8. *Frozen sections of Pipelle endometrial sections. Single labelled.* a) *LH+7, p-selectin (x40);* b) *LH+7, e-selectin (x40);* c) *fibronectin (x20).*

Table 6.11. *Endometrial E-selectin expression. LH+7 and LH+13.*

		LH+7			LH+13		
		Stroma	**Glandular epithelium**	**Surface epithelium**	**Stroma**	**Glandular epithelium**	**Surface epithelium**
NF	n	4	4	4	4	4	3
	Mean	1.00	1.13	1.00	0.88	0.88	1.00
	Median	1.00	1.00	1.00	1.00	1.00	1.00
	range	1-1	1-1.5	1-1	0.5-1	0-1.5	0.5-1.5
NI	n	9	9	7	5	6	6
	Mean	0.89	1.00	0.71	1.00	1.75	1.42
	Median	1.00	1.00	0.50	1.00	2.00	1.50
	range	0-1	0-2	0-2	1-1	1-2	0.5-2
PF	n	7	7	6	8	8	8
	Mean	1.00	1.43	0.67	0.94	1.38	1.36
	Median	1.00	1.50	1.00	1.00	1.00	1.00
	range	1-1	1-2	0-1	0-1.5	1-2	1-2
PI	n	9	9	9	4	4	4
	Mean	0.89	1.33	0.89	1.00	1.25	0.63
	Median	1.00	1.00	1.00	1.00	1.00	0.63
	range	0-1	0-2.5	0-2	1-1	1-2	0-1

Fibronectin

Fibronectin expression was strong (score 4) throughout the endometrial stroma including the endothelium (Fig. 6.8). There was no glandular or surface epithelial expression of fibronectin and expression was the same in all four groups and between LH+7 and LH+13.

Discussion

Several groups have considered the role of integrins and other adhesion molecules in endometrial function. Most widely published is the work of Lessey et al., most importantly in relation to α_1, $\alpha_v\beta_3$ and α_4 antigens. They have described the distribution of these antigens in defining the "window of implantation" (Lessey et al. 1994) and have suggested that in some subfertile women there is a failure to express these endometrial integrins in a normal pattern (Lessey 1995). This was related in

particular to so-called "out of phase" endometrium and associated failure of epithelial progesterone receptor down regulation (Lessey et al. 1996a).

In endometrial stroma, Lessey (1994) described expression of the α_1 antigen throughout the menstrual cycle, maximal during the proliferative phase and from the mid-secretory phase onwards with only low levels in the first part of the secretory phase. VLA-1 was present in epithelial cells only in the secretory phase. This distribution of α_1 expression is confirmed by current data. Similarly the present study has confirmed the distribution of $\alpha_v\beta_3$ in human endometrium which was reported by Lessey et al. (1994) with a general increase in expression in the late secretory phase. There was also a general decrease in α_4 expression at this stage of the menstrual cycle. Although both Lessey (1992) and Tabibzadeh (1992) suggested that α_4 was present only in epithelial cells, the present study has clearly shown VLA-4 expression throughout the stroma also, more in keeping with the report from Lessey et al. (1994). Klentzeris et al. (1993) did not find a significant cyclical change in VLA-4 either but did report a difference between fertile and subfertile endometrium, the subfertile tissue expressing little or no glandular epithelial VLA-4 and only low levels of expression elsewhere in contrast to fertile tissue where expression was detected throughout the endometrium and at all stages of the luteal phase of the menstrual cycle.

Lessey (1996) reported aberrant $\alpha_v\beta_3$ expression in out of phase endometrium with failure to produce the day 19 rise demonstrating an absence of β_3 in endometrium.

These findings were not reflected in the current study where neither fertility nor previous parity appeared to exert an effect on expression of this integrin.

Tabibzadeh and Poubouridis (1990) examined ICAM-1 expression in human endometrium. ICAM-1 was expressed uniformly throughout the endometrium and throughout the menstrual cycle, although more strongly in endothelial cells than either stromal or epithelial cells. This current study showed little or no expression in epithelium compared with stroma but confirmed the high endothelial expression. Tawia et al. (1992) suggested that there was no glandular or secretory epithelial expression of ICAM-1 which is more in keeping with the results described here.

Contrary to the report of Bilalis et al. (1996), fibronectin was clearly detected in the stroma and vessels of both fertile and subfertile endometrium thus challenging their interpretation that altered integrin expression may have a secondary effect on the secretion of extra-cellular matrix (ECM) proteins and thus affect implantation. They found a similar lack of collagen IV and laminin in subfertile tissue. These ECM proteins have not been re-examined in this current study.

Some differences in results reported by different groups may be related to the use of different monoclonal antibodies and also the possibility of cross labelling because of the shared subunit structure of some adhesion molecules. In addition methods of assessing the degree of expression of antigens in endometrium and its constituents varies between studies and different techniques may confer different sensitivities.

Conclusion

There are clear cyclical changes from LH+7 to LH+13 for some adhesion molecules, namely CD49α, CD61 and VLA-4. There appears to be little difference between the fertile and subfertile populations studied.

CHAPTER 7

THE EFFECTS OF EMBRYO DERIVED FACTORS ON ENDOMETRIAL LEUCOCYTES

THE EFFECTS OF EMBRYO DERIVED FACTORS ON ENDOMETRIAL LEUCOCYTE FUNCTION.

Introduction

From the preceding chapters and work by previous authors a complex picture of the potential roles of endometrial compartments and their products is emerging. It is likely that different areas of the endometrium such as epithelium and stroma are functionally discrete e.g. epithelium and stroma, although capable of interaction. There is clearly an overall control by ovarian steroid hormones but this is not necessarily by direct action on their effectors (Chapter 4).

Although cyclicity in several areas of the endometrium has been demonstrated there is less agreement on the putative deficiencies in subfertile women. In this study the fundamental difference in oestrogen and progesterone receptor status has been demonstrated in the endometrium of subfertile women (Chapter 3). It has not been possible however, to show a significant difference in potential downstream factors in these groups, neither leucocytes nor adhesion molecules differing significantly (Chapters 5 and 6).

It is possible that the endometrium is less dynamic in a non-pregnancy cycle than in the cycle where implantation is to occur. Its role after all is to provide a source of nourishment and protection for a successfully implanting embryo. The findings of the preceding chapters may have been coloured by the influence of late secretory changes, which in the absence of an embryo may be of less relevance to the functions

associated with implantation. These changes may represent instead the deterioration of unsupported tissue heading for menstruation.

In a conception cycle the endometrium is exposed not only to circulating hormones and endogenous soluble factors but also to a wide range of potential external effectors. Although much of the semen accompanying sperm does not enter the uterus, there are a large number of very potent effectors in its composition (Jequier 1995) and it is likely that some of these reach the endometrial cavity. Perhaps there are factors that influence endometrial function amongst them. It would seem reasonable that the fine tuning of endometrium is not required in a cycle where intercourse has not occurred. There is a suggestion that in mice seminal TGFβ1 is responsible for a transient inflammatory response in the endometrium following mating which may affect the maternal immune response to the pre-implantation embryo (Robertson et al. 1997). It is of note however, that successful embryo transfer after IVF is in general not preceded by insemination.

Of greater importance when considering the influence of "external" factors on endometrial behaviour is the embryo / endometrial interaction. Potential embryo derived products have been examined to establish a connection with endometrial receptivity and Nieder et al. (1987) demonstrated the secretion by mouse blastocysts of an array of 23 proteins *in vitro*. Lea et al. (1991) identified the polyamines spermine and spermidine in the pooled supernatants of human transfer grade embryos. They suggested that the presence of these molecules, conferring immunosuppressive activity to the supernatant, was required for successful

implantation. Sheth et al. (1991) measured IL-1 and IL-2 levels in embryo supernatants using commercially available ELISA kits and reported lower IL-1 levels in supernatants derived from women who subsequently achieved a pregnancy. The usefulness of this study was limited by the fact that embryos were cultured in groups and the supernatants from sibling embryos pooled prior to analysis. Thus no distinction could be made between individually successful or unsuccessful embryos and no account could be taken of the potential effect of one embryo on another. Seifer et al. (1993), in an attempt to overcome these limitations, performed ELISAs on supernatants of individual embryos cultured in 2mls of medium. They failed to demonstrate the presence of IL-6, IL-1α, IL-1β or leukaemia inhibitory factor (LIF) in these culture supernatants, possibly because the concentrations were lower than the sensitivity of the ELISA kits. In all these studies culture supernatants were harvested at the time of embryo transfer, 2-3 days from fertilisation. In 1995 Austgulen et al. reported the presence of IL-1, IL-6, TGFβ and receptors to TNF but no TNF in the culture supernatants of embryos derived from IVF. There was no correlation with embryo morphology. Simón et al. in 1997 described the examination of individual embryo culture supernatants for IL-1 but did not report their findings.

The direct examination of embryo culture supernatants has not advanced significantly and the effects of known embryo derived proteins on the endometrium itself has been highlighted for consideration. Work in mice has drawn attention to LIF as an important factor in implantation, focussing attention on this cytokine in the human system. Several groups (Charnock-Jones et al. 1994, Kojima et al. 1994;

Arici et al. 1995; Cullinan et al. 1996) have shown cycle dependent expression of LIF in human endometrium, a cytokine which in mice is an absolute requirement for fertility (Stewart et al. 1992). Expression of the receptor for this factor has been inferred from the finding of blastocyst messenger ribonucleic acid (mRNA) for the LIF receptor (LIF-R) at the appropriate stage of development (Charnock-Jones et al. 1994). Roles for this potential embryo/endometrial interaction include effects on trophoblast differentiation (Bischoff et al. 1995, Nachtigall et al. 1996). In addition, recent work by Simón et al. (1993, 1994) has greatly increased the understanding of the "IL-1 system" in humans, investigating the distribution in endometrium and embryo of the components of that system (IL-1α, IL-1β, IL-1R types I and II and IL-1R antagonist [IL-1Ra]). This group has shown that the secretion of IL-1α and IL-1β by human embryos is fundamental to their ability to adhere to human endometrium *in vitro* (Simón et al. 1997). This appears to be the result of up-regulation of endometrial β_3 integrin expression.

Embryo / endometrial interactions in humans have been reviewed extensively (Simón and Pellicer 1995; Simón et al.1999). However, the lack of animal models in which to study the processes of human invasive implantation and the regulated and restricted used of human embryos in research (Human Fertilisation and Embryology Act 1990), means that additional novel methods for investigating these interactions are required.

In the Centre for Reproductive Medicine, Royal Victoria Infirmary during the time of this study there was no freezing programme for spare embryos produced following IVF. Consequently there was a significant number of embryos which were either discarded or used in licensed research programmes. Embryos, which are to be discarded, may, under the Human Fertilisation and Embryology Act (1990) be maintained in-vitro for no longer than 14 days following fertilisation. The value of prolonged culture performed routinely is to assess blastocyst development (Fig. 7.1). Crude information derived from this process is used in the assessment of couples for future cycles of treatment as blastocyst development is considered to reflect good quality embryos. A couple failing to conceive in a particular cycle of IVF treatment, but producing a significant number of blastocysts in spare embryos, despite the "best" ones having been replaced, would be considered to be at an advantage over a couple where blastocyst development was poor. Embryo quality would be less of a deciding factor in their chances of a successful cycle of treatment in any subsequent cycle.

In a research setting the advantage of collecting culture supernatants from such embryos is that although pregnancy outcome for each embryo cannot be assessed, the potential success of each embryo can be ascertained by its development or otherwise to blastocyst stage. In contrast in the studies described above, embryo culture supernatants were harvested at the time of embryo transfer. In addition the success of an individual embryo cannot be inferred from pooled culture supernatants since the contribution of a single embryo cannot be determined and the triplet rate - 100% implantation rate - is only around 5% for a 3 embryo transfer. As individual cultures

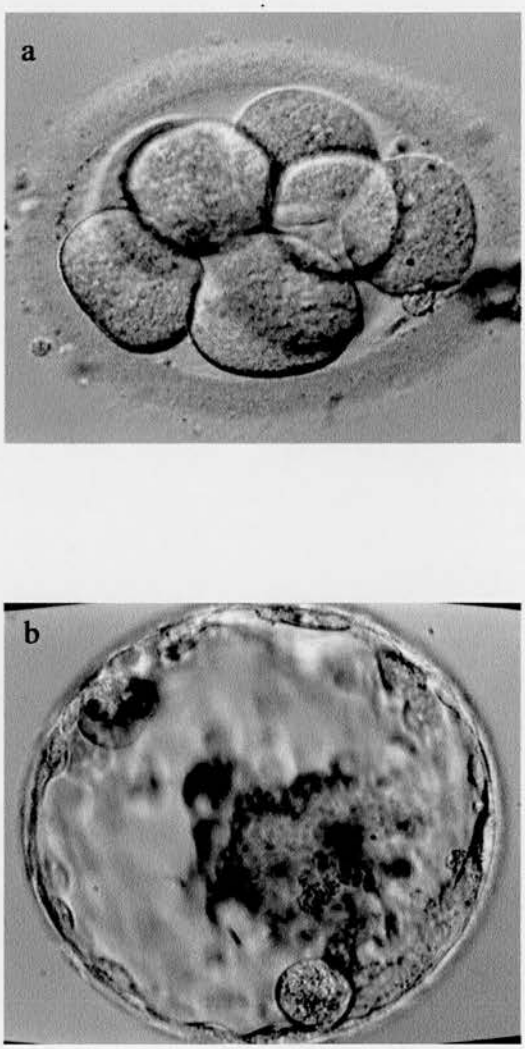


Figure 7.1. a) *8-cell human embryo,* b) *human blastocyst.*

are performed in relatively low volumes in this laboratory and culture for these purposes was for 5 days, then it is possible that higher concentrations of any embryo products would be established by the time of harvest and analysis.

In considering endometrial leucocyte function it is of value to investigate the potential effect of embryo derived products on these individual populations. The tiny volume of supernatants available requires development of low volume assays.

It is with the interest in endometrial leucocytes and their functions underlying this thesis, that methods have been investigated to examine the effect of embryo derived factors on individual leucocyte populations isolated from endometrium.

1. EVALUATION OF A LOW VOLUME LEUCOCYTE PROLIFERATION ASSAY

As has been discussed, in order to evaluate the properties of individual embryo culture supernatants, any assay must make use of relatively small volumes of material. In order to examine the effect of such culture supernatants on endometrial leucocyte populations, also available in limited numbers, small volume leucocyte functional assays are required. In the following study peripheral blood lymphocytes have been used in such a low volume assay. The aim was to evaluate the technique.

Standard mitogen-induced peripheral blood leucocyte proliferation assays are performed in 96-well, flat-bottom microtitre plates (Nunclon, Paisely, UK) (Fig. 7.2a) requiring $1x10^5$ cells per well in a final volume of 200µl. The smaller volume 60 well Terasaki plates (Nunclon) (Fig. 7.2b) require around $4x10^3$ cells per well in a

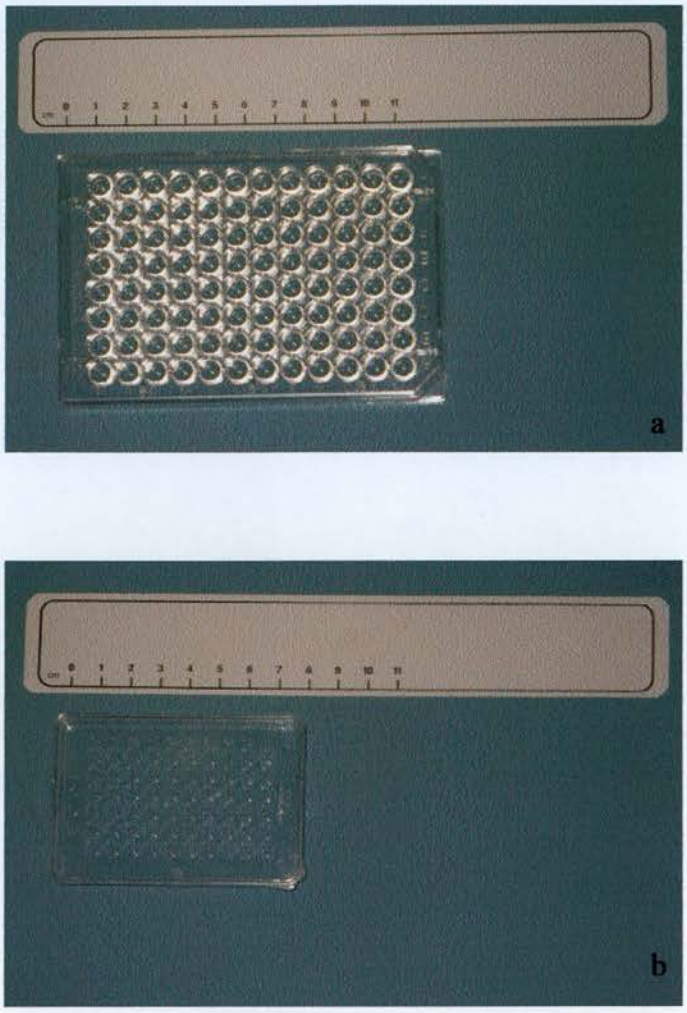


Figure 7.2a) *Standard 96-well plate.* b) *Terisake plate. Scale is in centimetres.*

hanging drop of 20µl final volume. This part of this chapter outlines the evaluation of such low volume assays for proliferation of peripheral blood lymphocytes.

Materials and methods

Preparation of peripheral blood lymphocytes

20mls of blood was obtained at venepuncture and immediately heparinised (20iu/ml) in a universal container. An equal volume of phosphate buffered saline (PBS, pH 7.4) was added to the blood and mixed. In universal containers, 10ml aliquots of diluted blood were each layered onto 5mls of Lymphoprep TM (Nycomed Ltd., Oslo, Norway) producing a sharp interface. These preparations were centrifuged at 800G for 20 minutes without the brake. The resulting intermediate cloudy layer was harvested from the four tubes and each added to 25mls of PBS. Further centrifugation was performed (400G; 15 minutes; 3 brake). Each pellet was resuspended in 1ml PBS and topped up to 25mls. Centrifugation was performed again (200G; 10 minutes; 3 brake). Each pellet produced was resuspended in 1ml RF10 (incomplete RPMI medium supplemented with 10% fetal calf serum [Appendix 4]) for a cell count to be performed. This suspension was further diluted to give an appropriate cell concentration.

Proliferation assay

Assays were set up using standard 96 well plates and the smaller 60 well Terasaki plates. Triplicate or quadruplet wells were used and cells were incubated with phytohaemagglutinin (PHA, Sigma) at varying concentrations to stimulate proliferation. In the 96-well plates 100µl of mitogen was added to 100µl PBLs in

RF10 ($1x10^5$ cells/well) to achieve a final total volume of 200µl whilst in the Terasaki plates 10µl of mitogen was added to 10µl of PBLs in RF10 ($4x10^3$ cells/well). The plates were incubated for three days at 37°C in humidified 5% CO_2. Terasaki plates are inverted for all incubations, producing a "hanging drop" culture.

Controls

Control wells, used in each experiment described below, contained either cell preparations with medium alone substituting for PHA, or medium containing no cells replacing the cell preparation. In addition there were wells containing neither cells nor PHA, both having been replaced by medium.

Pulsing

Tritiated (^3H) thymidine (Amersham Life Sciences, Little Chalfont, UK) was added to each well, 0.4µCi/well to the 96-well plates (10µl of 40µCi/ml stock solution) and 0.16µCi/well to the Terasaki plates (1µml of 160µCi/ml stock). Plates were then incubated for a further six hours before harvesting.

Harvesting

For 96-well plates, the plate was harvested by aspiration onto filters which were dried overnight prior to counting with a Direct Matrix β Counter without scintillation fluid.

For Terasaki plate vacuum harvesting onto filters from the inverted plate was performed using equipment designed specifically for this purpose (Flow Labs). The

filters were dried overnight and individual discs combined with 20µl liquid scintillant (Microscint-O™, Packard, Canberra, Australia) on a microplate scintillation counter (Top Count™, Packard).

Experimental design

Experiment 1 - 96-well plates compared with Terasaki plates

This experiment was performed to compare peripheral blood leucocyte proliferation assays in the two plate types using different concentrations of PHA. Triplicate wells were set up in each plate as described above. PHA concentrations of 2µg/l, 1µg/l and 0.5µg/l were used. Controls were set up as described.

Experiment 2 - Optimisation of PHA concentration

This experiment was designed to assess the optimal concentration of PHA to induce PBL proliferation in Terasaki plate assays. PHA concentrations of 4µg/l, 2µg/l and 1µg/l were assessed in triplicate wells as described above.

Experiment 3 - Optimisation of cell concentration

This experiment confirmed the ideal PHA concentration to be used by examining doubling dilutions from 8µg/l to 0.125µg/l. This was combined with an assessment of the most useful cell concentration to use in the Terasaki plates. A higher cell concentration was proposed because of relatively low counts achieved in some of the preliminary experiments. It would be predicted that a higher cell concentration would achieve a higher stimulation index (SI) but very high cell concentrations may outstrip a well's capacity to support the proliferating culture and therefore result in a

lower SI. The experiment was performed with cell concentrations giving $4x10^3$ cells/well and $1x10^4$ cells/well.

Experiment 4 - The effect of conditioned embryo culture medium (EC medium)

This experiment tested the effects of a range of mixtures of RF10 and EC medium [Appendix 4] media (100/0 to 50/50) and 100% EC medium on the above proliferation assay. This was done to exclude the possibility that EC medium in the embryo culture supernatants to be assessed would have direct effects on cell proliferation. EC medium was "conditioned" prior to use by storage in the embryo incubator (5% CO_2) for 24 hours under the same conditions as the embryo cultures (see below).

Results

In the results of each of the experiments outlined below initial data are expressed in mean counts per minute (cpm) of either 3 or 4 replicate wells. These values however, do not allow direct comparisons to be made between different experiments performed on different occasions. In these experiments stimulation index (SI) relates to the effect of added mitogen and is defined as the mean cpm from test wells in the presence of PHA / mean cpm for control wells with cells and medium alone, added. This allows for better comparison between experiments.

Thus the following equation was employed:-

$$SI = \frac{\text{cpm test wells [cells + PHA]}}{\text{cpm control wells [cells + no PHA]}}.$$

Experiment 1

The results obtained using Terasaki plates in this assay were equivalent to those obtained using 96 well plates on a qualitative basis (Table 7.1). Direct comparison of counts cannot be made because of the different techniques and equipment used. Stimulation index (SI) was calculated for both plates to compare the two (Table 7.1). Since there is a possibility of synergism between cells in culture, conditions in the hanging drops of Terisake plates may differ from those of the larger 96-well plate with bigger cell numbers. The pattern of stimulation between the two plate types is similar even though actual stimulation index values appear lower (5.4 – 17.9) when compared with the 96-well plates (26.3 – 56.6). This indicates that use of hanging drop Terasaki plates is a useful approach to assessing leucocyte function with the limited numbers of lymphocytes likely to be obtained, for example, from Pipelle samples.

Table 7.1. *Comparison of 96-well versus Terasaki plates.*

PHA concentration	**2µg/l**	**1µg/l**	**0.5µg/l**	**0µg/l**
96-well				
median cpm.	10269	5152	4813	177
range	10065-10269	4570-7197	4462-5181	173-199
SI	56.6	30.8	26.3	0
Terasaki				
Median cpm	1113	682.5	333.9	63.2
range	1094.7-1131.3	491.6-932.6	303.4-364.4	49.4-74.5
SI	17.9	11.3	5.4	0

Experiment 2

In order to optimise the experimental conditions used in the Terasaki plates, the range of PHA concentration used was widened. In this situation 2μg/l gave a higher optimal SI (43.7) than either 4μg/l (SI=21.1) or 1μg/l (SI=39.7) (Table 7.2).

Table 7.2. *Comparison of PHA concentrations in Terasaki plate*

PHA concentration	**4μg/l**	**2μg/l**	**1μg/l**	**0μg/l**
median cpm	1786.6	3682.4	3354.6	103.2
range	1613.9-1959.3	3229.3-4186.2	3331.4-3377.7	46.2-104.3
SI	21.1	43.7	39.7	0

Experiment 3

Again in order to optimise experimental conditions in the Terasaki plates two PBL concentrations were assessed over a range of PHA concentrations. Higher stimulation indices were achieved using a higher concentration of cells as was predicted (maximum SI 23.6 [PHA 4μg/l]) compared with the lower concentration (maximum SI 11.4 [PHA 2μg/l]). Thus a useful working cell concentration of $1x10^4$ cells/well for use in subsequent assays was demonstrated (Table 7.3). Since the primary objective was to establish an assay useful for the assessment of the immunoregulatory effect of embryo supernatants on PHA induced leucocyte proliferation, the capacity for further stimulation in the hanging drop cultures needs to be allowed for. The maximum SI achieved by $1x10^4$ cells was at a PHA concentration of 4μg/l, whilst increasing the PHA concentration to 8μg/l, reduced the SI. This may be the result of saturation of the response, a separate inhibitory or even

Table 7.3. *Comparison of cell concentrations in Terasaki plate*

	$4x10^3$ cells/well			**$1x10^4$ cells/well**		
PHA concentration	Median cpm	Range	SI	Median cpm	range	SI
8µg/l	570.6	418-675	5.6	1223.9	1064-1167	14.1
4µg/l	682.5	488-877	6.8	2009.3	1832-2195	23.6
2µg/l	1103.0	1034-1294	11.4	1750.5	563-2120	17.4
1µg/l	1030.7	833-1194	10.2	1370.2	1228-1628	16.5
0.5µg/l	465.4	356-664	4.9	1020.6	428-1186	10.3
0.25µg/l	313.85	230-365	3.1	332.9	146-520	3.9
0.125µg/l	190.7	126-371	2.2	112.3	83-177	1.4
0µg/l	97.3	32-170	0	82.3	57-119	0

toxic effect of higher concentrations of mitogen, or the ability of the culture to outgrow the nutritional resource of the well such that further expansion cannot be supported. Thus in order to allow scope for additional stimulation of proliferation in the presence of embryo supernatants, 2µg/l PHA was considered a suitable concentration for use in future assays.

Experiment 4

The aim of these experiments was to evaluate the small volume leucocyte proliferation assay for use with endometrial leucocytes available in restricted cell numbers and embryo culture supernatants available in restricted volumes. Embryos are cultured in a specialised medium. This experiment was designed to test the effect of conditioned embryo medium on PHA induced PBL proliferation. A range of ratios of conditioned embryo culture medium with RF10 was assessed (0/100 to 50/50 volume/volume). No titratable effect was seen (mean cpm 2874.6 – 3900.2) confirming that the presence of conditioned embryo culture medium had no consistent effect on PHA induced peripheral blood cell proliferation (Table 7.4).

Table 7.4. *Effect of EC medium on cell proliferation.*

4μg/ml PHA	**median cpm**	**range**
RF10/EC medium ratio		
100/0	3002.8	1280-7031
90/10	3039.3	1534-3886
80/20	3281.8	2974-3868
70/30	4503	2384-4814
60/40	3203.6	2723-3846
50/50	3050.2	2461-3428

Conclusion

With reference to the optimisation of experimental conditions, it can be seen that the use of 60 well Terasaki plates is appropriate for the examination of leucocyte proliferation using low cell numbers. An optimal working cell concentration of $1x10^4$ cells per well and a PHA concentration of 2μg/ml were used. In addition it has been shown that the presence of conditioned embryo culture medium alone has no effect on the PHA induced peripheral blood lymphocyte proliferation.

2. Assessment Of The Effect Of Individual Embryo Culture Supernatants On PHA Induced Peripheral Blood Leucocyte Proliferation.

The second section of this study addresses the potential to assess the effects of single embryo culture supernatants in small volume leucocyte assays. Following the evaluation of the use of Terasaki plates in PHA induced peripheral blood leucocyte proliferation assays it can be seen that such assays could be used with limited volumes of test culture supernatants. These supernatants potentially contain embryo derived factors involved in successful embryo-endometrial interactions.

Oocytes retrieved for the process of IVF are mixed with the sperm preparation soon after collection. 16-20 hours later they are checked for fertilisation. In the presence of normal eggs and sperm a fertilisation rate of approximately 70-80% is expected. Embryo replacement is usually performed two days following collection. At the time of this study, in this unit spare embryos are cultured in 100μl embryo medium in droplets containing three or more. These were routinely grown on to blastocyst stage then discarded. For the experiments to assess the effect of individual embryo culture supernatants, the technique was modified to culture single spare embryos in 100μl droplets. The culture supernatants of these individual embryos were harvested at the time of blastocyst disposal when the low volume of each supernatant was likely to contain early gene products secreted during the normal growth of the embryo.

Materials and methods

Embryo and blastocyst supernatants

In this unit it was normal practice to set up multiple embryo culture of spare embryos for growth to blastocyst stage as discussed above. For the purposes of this study, single embryo cultures were set up when, after embryo transfer, there were approximately 10 spare embryos available and where it was expected from a couple's previous history that a "good" blastocyst response would be achieved in those embryos. Embryos were maintained in 100μl droplets in EC medium [Appendix 4] at 37°C and in 5% CO_2. For each set of sibling embryos, supernatants from both blastocysts and less well developed embryos were harvested when the embryos themselves were discarded (at around day 12). The culture supernatants were immediately frozen and stored at –70°C.

Peripheral blood leucocyte preparation

This was performed as described above. The PBL cell concentration was adjusted to $2x10^6$ cells/ml.

Proliferation assays

60 well Terasaki plates were used and to each well was added 5µl of PBLs ($1x10^4$ cells/well), 5µl PHA (to give final concentration of 2µg/ml) and 10µl of embryo culture supernatant. Quadruplet wells were set up. Incubation, pulsing and harvesting were performed as described above.

Controls

Multiple control wells included those where either no cells or no PHA was added in the presence of the other components with extra medium (RF10) making up the volume. As a control for the effect of culture supernatant, wells contained conditioned embryo culture medium.

Evaluation

Plates were analysed as described above giving a value of counts per minute (cpm) for each well. The stimulation index relating to the effect of embryo culture supernatants on PHA induced PBL proliferation was calculated using the following equation:-

$$SI = \frac{\text{cpm test wells [cells + PHA + embryo culture supernatant].}}{\text{cpm control wells [cells + PHA + conditioned embryo medium]}}$$

This SI therefore represents only the effect of the presence of the embryo culture supernatant on PHA induced PBL proliferation. Thus a figure for SI less than 1 denotes suppression whilst a figure greater than 1 denotes stimulation.

In addition the stimulation index for the effect of embryo culture supernatant on PBL proliferation in the absence of mitogen was calculated thus:-

$$SI = \frac{\text{cpm test wells [cells + embryo culture supernatant].}}{\text{cpm control wells [cells + conditioned embryo medium]}}$$

In each case the percentage inhibition was calculated using the following:-

$$\% \text{ inhibition} = (1 - SI) \times 100$$

Statistical analysis

Comparisons of embryo types (blastocysts versus less well developed embryos) and the effects of their culture supernatants on PHA induced PBL proliferation, were assessed using the Mann-Whitney test of significance.

Results

The effects of embryo culture supernatants on PHA induced proliferation of peripheral blood lymphocytes was heterogeneous but generally indicated a reduction in the level of proliferation.

The percentage inhibition with supernatants from blastocysts ranged from -48.8 (ie. no inhibitory effect) to +83.4 (ie. inhibitory effect) with PHA (Table 7.5) and -76.8 to +72.9 without PHA (Table 7.6). In the case of non-blastocyst culture supernatants the ranges were –119.9 to +58.9 with PHA (Table 7.5) and –142.3 to +61.3 without PHA (Table 7.6). These wide ranges of effects are reflected in the heterogeneity, not only between embryos of different subjects but also between sibling embryos. Figure 7.3 shows the broad scatter of values of percentage inhibition for each group of embryos tested.

Table 7.5. *The effects of individual embryo culture supernatants on PHA induced peripheral blood lymphocyte proliferation.*

Subject	**Embryo**	**SI**	**% inhibition**	**Blastocyst**	**SI**	**% inhibition**
BK	1	0.4	58.9	1	1.0	3.3
	2	0.7	26.4	2	0.6	44.3
				3	*1.1*	*-6.9*
JH	1	0.5	50.0	1	0.8	23.2
	2	*1.2*	*-21.5*	2	0.7	27.7
	3	0.7	30.9	3	0.2	81.8
	4	1.0	3.8	4	*1.5*	*-48.8*
LC	1	0.9	6.9	1	*1.0*	*-1.1*
	2	*1.0*	*-2.3*	2	0.8	22.7
JC	1	*2.2*	*-119.9*	1	0.5	52.3
	2	*1.6*	*-61.2*	2	0.7	33.1
				3	0.9	12.2
				4	0.9	12.4
				5	*1.5*	*-48.7*
KS	1	*1.1*	*-12.0*	1	*1.2*	*-14.7*
	2	*1.1*	*-7.4*	2	*1.1*	*-14.3*
JHB	1	0.7	31.9	1	0.7	30.8
	2	*1.3*	*-30.2*	2	0.8	21.9
	3	0.9	11.1	3	0.5	46.4
AB	1	0.9	7.7	1	0.6	35.3
GH	1	*1.2*	*-18.4*	1	0.2	83.4
	2	*1.5*	*-50.4*	2	0.7	28.6

Figures in italics denote stimulation.

Table 7.6. *The effects of individual embryo culture supernatants on peripheral blood lymphocyte proliferation without PHA.*

Subject	Embryo	SI	% inhibition	Blastocyst	SI	% inhibition
BK	1	0.8	19.4	1	0.4	62.8
	2	0.4	61.3	2	*1.3*	*-28.9*
				3	0.3	66.9
JH	1	0.8	24.7	1	0.6	42.1
	2	0.6	44.4	2	*1.8*	*-76.8*
	3	0.9	9.2	3	0.9	10.4
	4	0.5	45.9	4	0.4	56.1
LC	1	0.7	30.8	1	0.3	68.2
	2	0.5	50.4	2	0.3	73.0
JC	1	0.4	57.5	1	*1.2*	*-19.7*
	2	*1.1*	*-8.7*	2	0.3	70.1
				3	0.7	30.3
				4	0.5	48.5
				5	0.4	65.0
KS	1	0.9	5.3	1	0.5	47.5
	2	*2.4*	*-142.3*	2	0.4	58.3
JHB	1	0.9	13.6	1	*1.3*	*-26.8*
	2	0.6	44.0	2	0.4	61.3
	3	0.6	43.9	3	0.4	60.6
AB	1	*1.1*	*-7.0*	1	*1.1*	*-6.7*
GH	1	0.9	11.3	1	0.3	70.1
	2	0.7	30.1	2	0.3	72.9

Figures in italics denote stimulation.

The data described above were collected in three separate experiments. The PHA stimulation indices for these experiments, that is, the effect of PHA on PBL proliferation in the absence of embryo culture supernatant ranged from 5.2 to 39.4. This illustrates the need for conversion of counts per minute to stimulation index in order to make useful comparisons between assays performed on different occasions.

Blastocyst versus non-blastocyst

Although both groups, blastocyst and non-blastocyst culture supernatants had an inhibitory effect on peripheral blood lymphocyte proliferation, there were no significant differences shown between the blastocyst and non-blastocyst experiments either with ($p=0.446$) or without PHA ($p=0.550$) (Table 7.7).

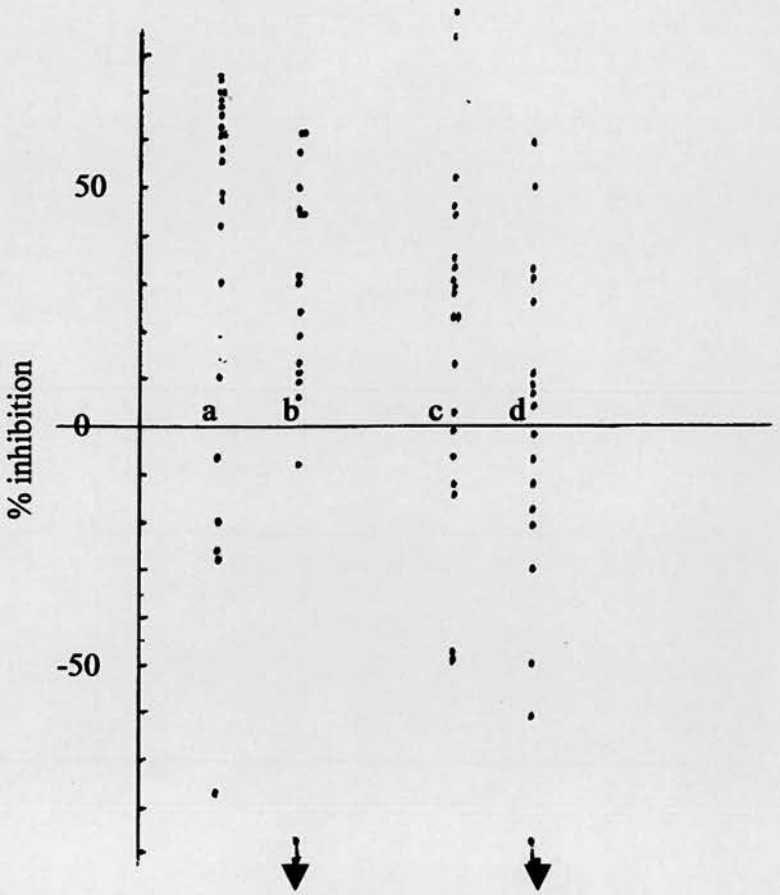


Figure 7.3. *Scatter of inhibition (and stimulation - negative plots) of peripheral blood lymphocyte proliferation without PHA;* a) *blastocyst and* b) *non-blastocyst supernatants and with PHA;* c) *blastocyst and* d) *non-blastocyst supernatants.*

Table 7.7. *Percentage inhibition of peripheral blood lymphocyte proliferation by embryos.*

Without PHA	**Median % inhibition**	**range**	**Significance**
Blastocyst	57.2	-76.8 to +73.0	0.446
Non-blastocyst	27.4	-142.3 to +61.3	
With PHA			
Blastocyst	22.9	-48.8 to +83.4	0.550
Non-blastocyst	0.7	-119.9 to +58.9	

Mann-Whitney test. Significant if $p \leq 0.05$.

The effect of PHA

Despite the lack of a significant difference between blastocyst and non-blastocyst supernatants on PBL proliferation, it can be seen that in both groups the inhibition of proliferation was less when PHA was present. This was significant in the blastocyst group (p=0.047) (Table 7.8).

Table 7.8. *Percentage inhibition of peripheral blood lymphocyte proliferation by embryos. The effect of PHA.*

Blastocysts	**Median % inhibition**	**Range**	**Significance**
Without PHA	57.2	-76.8 to +73.0	***0.047***
With PHA	22.9	-48.8 to +83.4	
Non-blastocysts			
Without PHA	27.4	-142.3 to +61.3	*0.098*
With PHA	0.7	-119.9 to +58.9	

Mann-Whitney test. Significant if $p \leq 0.05$.

Conclusion

The supernatants of cultured embryos appeared to be generally inhibitory although this was less clear when PHA was present. There appeared to be no difference in the inhibitory effect of supernatants from blastocysts on PBL proliferation when compared with supernatants from non-blastocysts but the presence or absence of PHA stimulation had a significant effect in the case of culture supernatants from

blastocysts. It can be seen however, that individual embryo supernatants varied widely in their regulatory effects on PBL proliferation and this did not appear to be associated with parental origin.

3. Isolation Of CD3 + Lymphocytes From Pipelle Endometrial Samples

The previous experiments in this chapter have established the usefulness of the small volume PHA induced PBL proliferation assay to assay embryo culture supernatants. In order to make such assays relevant to endometrial work evaluation using isolated endometrial leucocyte populations is required. In addition, it is preferable to avoid the cloning of cells such that normal function is compromised as little as possible. Isolation from endometrium of purified eGLs (98%) has been achieved in this laboratory by other workers (Jones et al. 1997c, Vassiliadou and Bulmer 1998). Their function in relation to time of cycle, miscarriage and endometriosis have subsequently been investigated *in vitro* without cloning. In these situations however, endometrium from a major proportion of the cavity of the hysterectomised uterus or a sizeable portion of early pregnancy decidua has been available as a source of cells and as previously noted eGLs comprise the greatest proportion of the endometrial leucocyte population.

For work relevant to fertility, timed endometrial samples are required. Hysterectomy specimens are therefore rarely of value since when performed in young women, the indication either far outweighs any research needs for timing, for example cervical cancer, or may have an endometrial component which could affect the results, for example endometriosis. Timed endometrial biopsies are more appropriate in this

setting as previously discussed but the numbers of leucocytes available are likely to be correspondingly small.

In the final part of this chapter T cells, comprising the smallest, yet a potentially important endometrial leucocyte population, have been isolated from a Pipelle sample of endometrium to confirm that adequate numbers of such cells can be retrieved by this technique for study in low volume assays. This would allow assessment of their functions *in vitro* without the need to expand their numbers prior to functional assays.

Materials and methods

Endometrial sampling

A Pipelle endometrial biopsy was taken from a uterus obtained at hysterectomy in the secretory phase for non-endometrial causes. The technique used to obtain the sample mimicked that performed *in vivo* [Appendix 2].

Isolation of CD3+ lymphocytes.

The endometrial sample was finely minced and digested in 0.1% collagenase type II (Sigma Chemical Co.) and 0.01% DNAase type IV (Sigma Chemical Co.) in incomplete medium (Appendix 4) at room temperature for 40 minutes. The digest was sieved through a 40μm cell strainer and then treated with 0.84% ammonium chloride for a further 10 minutes to lyse red cells. Cells were then washed in incomplete medium. Nigrosin staining of a small sample confirmed good viability. The sample was then resuspended in RF10 for a cell count and adjusted to a

concentration of approximately $1x10^6$ cells/ml. Plastic adhesion to remove macrophages and stromal cells was achieved with overnight culture (5% CO_2, 37°C) in a $75cm^3$ plastic culture flask, and non-adherent cells retrieved, namely lymphocytes.

For positive selection of T cells, isolated lymphocytes were resuspended in incomplete medium and incubated with primary FITC conjugated mouse anti-CD3 antibody (Becton Dickinson), used at 5µl / $1x10^6$ cells and diluted at 1:100 with incomplete medium, for 30 minutes at 4°C. This cell and antibody suspension was added to 20mls ice cold phosphate buffered saline pH 7.4 (PBS) and centrifuged (10 minutes, 200G). The resultant pellet was then resuspended in a further 20mls PBS. A final pellet was obtained by centrifugation (10 minutes, 200G). This pellet of cells was then resuspended in Mini MACS buffer containing 40µl of MACS IgG1 and IgG2 mixed microbeads beads and incubated for 15 minutes at 4°C. Mini MACS magnetic beads are coated with anti mouse IgG and thus bind the antibody labelled cells. After this period, the cells were adjusted to 1ml with Mini MACS buffer and dispersed using a 26G needle. The bead labelled cells were loaded onto a Mini MACS column in a magnetic field which retains them in the column, and washed with 500µl of Mini MACS buffer to wash out any remaining CD3 negative cells (fraction MM1) (Fig. 7.4). This fraction therefore should contain all the unlabelled cells. Further washes with 2 x 500µl of Mini MACS buffer were performed and the elutant, MM2, checked to ensure it did not contain cells. This fraction was discarded.

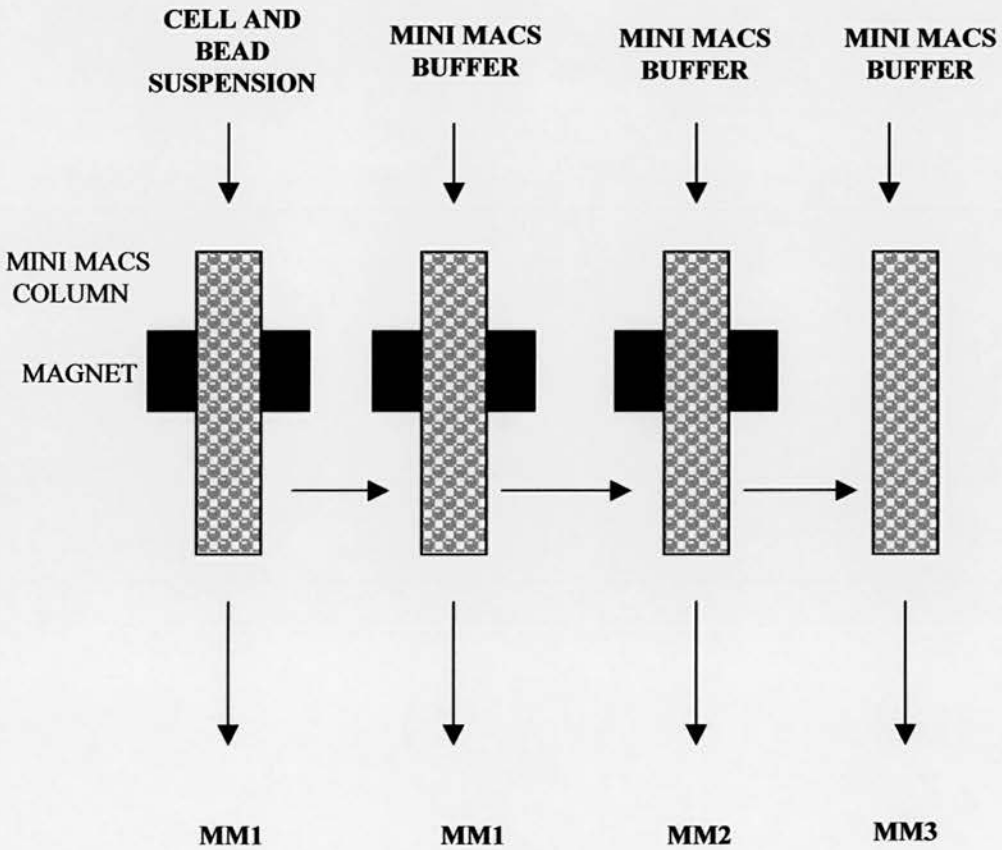


Figure 7.4. *Diagram of Mini MACS process. MM1 contains all cells not labelled with beads. MM2 contains no cells. MM3 contains CD3 positive bead-labelled cells.*

The column was then removed from the magnet, and washed 2 further times with 2 x 500µl of Mini MACS buffer to elute the CD3 positive cells into fraction MM3.

Evaluation

Flow cytometrical analysis (FACS) of MM1 and MM3 was performed to assess purity and characterise the cell populations represented in each. The samples were analysed immediately using a flow cytometer (FACScan, Becton Dickinson) and the "Cell Count" software (Becton Dickinson). Gates were set to known T cell values to exclude dead cells and debris. Histograms were plotted.

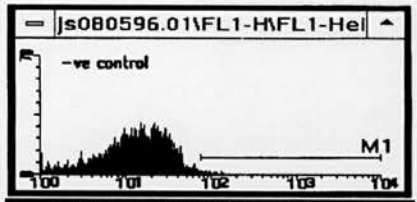
Results

Following digestion of the Pipelle endometrial sample $38x10^6$ cells were obtained in total with >95% viability. $15.25x10^6$ non-adherent cells were obtained after overnight culture.

Following elution from the Mini MACS column the MM1 contained $13.3x10^6$ cells ($6.65x10^6$/ml) whilst MM3 contained $0.625x10^6$/ml.

FACS analysis of the cell preparation prior to Mini MACS selection showed that 9.41% of the cells were CD3 positive. FACS analysis of the Mini MACS fractions confirmed the separation of two separate populations of cells. MM3 contained CD3 positive cells with a purity of 99.36% whilst MM1 contained only 3.28% CD3 positive cells (Figure 7.5).

a)

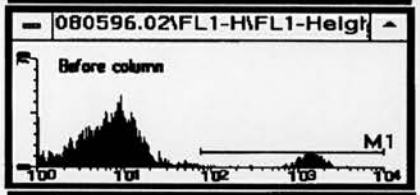


Js080596.01\FL1-H\FL1-Height

-- Geometric Histogram Statistics
SELECTED PREFERENCES: Geometric/Linear
Parameter FL1-H FL1-Height Gate G1- R1

M	Left,	Right	Events	%	Peak	PkChl	Mean	Median
0	1.00,	9646	1471	100.00	30	14.86	12.29	13.82
1	74.99,	9646	17	1.16	2	74.99	94.65	86.60

b)

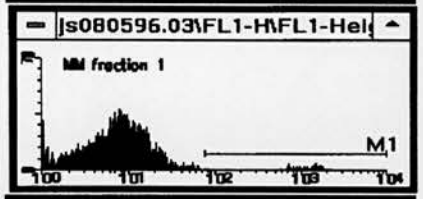


Js080596.02\FL1-H\FL1-Height

-- Geometric Histogram Statistics
SELECTED PREFERENCES: Geometric/Linear
Parameter FL1-H FL1-Height Gate G1- R1

M	Left,	Right	Events	%	Peak	PkChl	Mean	Median
0	1.00,	9646	1871	100.00	58	1.00	10.24	7.77
1	74.99,	9646	176	9.41	10	1286	1237.64	1382.37

c)

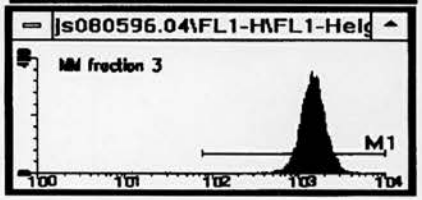


Js080596.03\FL1-H\FL1-Height

-- Geometric Histogram Statistics
SELECTED PREFERENCES: Geometric/Linear
Parameter FL1-H FL1-Height Gate G1- R1

M	Left,	Right	Events	%	Peak	PkChl	Mean	Median
0	1.00,	9646	1678	100.00	57	1.00	7.99	7.77
1	74.99,	9646	55	3.28	4	723.39	1092.33	1333.52

d)



Js080596.04\FL1-H\FL1-Height

-- Geometric Histogram Statistics
SELECTED PREFERENCES: Geometric/Linear
Parameter FL1-H FL1-Height Gate G1- R1

M	Left,	Right	Events	%	Peak	PkChl	Mean	Median
0	1.00,	9646	7056	100.00	357	1382	1401.62	1433.01
1	74.99,	9646	7011	99.36	357	1382	1443.23	1433.01

Figure 7.5. *Data from FACS analysis of* a) *negative control - preparation with CD3 positive cells removed,* b) *cell preparation prior to Mini MACS separation,* c) *Mini MACS fraction 1 (MM1) and* d) *Mini MACS fraction 3 (MM3).*

Conclusion

CD3 positive T cells could be isolated from digested Pipelle samples using the Mini MACS process in low numbers (~$60x10^4$ cells) suitable for low volume functional assays in Terasaki plates and at high purity (99.36%).

Discussion

The aim of this chapter was to consider the feasibility of examining the interactions of isolated endometrial leucocytes and embryo products. The present study has established that, whereas in previous studies pooled embryo culture supernatants were used (Sheth et al. 1991), small volume leucocyte functional assays, such as the PHA induced PBL proliferation assay, can be used to successfully assess the immunoregulatory effect of individual embryo culture supernatants. In addition, the present finding of heterogeneity observed amongst supernatants of blastocysts and non-blastocysts and between sibling embryos means that useful extrapolation of their *in vitro* behaviour to *in vivo* outcome using pooled or even individual culture supernatants is doubtful.

Although cloned isolated CD56 positive eGLs have previously been examined for function, it is only recently that their *in vitro* response to possible effectors has begun to be examined using isolated non-cloned cell populations. The smaller endometrial leucocyte populations have not previously been isolated and examined in this way. It has been shown in the present study that there are suitable techniques for their examination in small numbers; and moreover, these leucocytes may be isolated with high levels of purity from small endometrial biopsies in sufficient numbers for use in

these small volume assays. These findings, have not been elaborated further in this thesis but pave the way for further interesting research into the interactions between the embryo and the endometrial immunological response.

CHAPTER 8

DISCUSSION

DISCUSSION

The aim of this thesis was to try to elucidate endometrial features that may have a bearing on the problem of unexplained sub fertility. Around 1 in 7 couples attempting to achieve pregnancy will experience difficulties and of those approximately 25% will have entirely normal investigations. Sadly despite the great technological advances of recent years in the field of assisted reproductive treatment, there have been few inroads made in narrowing the diagnosis for this particular group of couples. It remains therefore that the best option for successful management of unexplained subfertility is IVF or GIFT. For couples whose chance of spontaneous conception remains at 1 to 3% per menstrual cycle (Hull et al. 1985), IVF and GIFT are not only hugely over-sophisticated treatments but they will only achieve 15-40% pregnancy rate per treatment cycle (Hull 1992). Since numerous factors may be implicated there is little precision in the use of ART in this situation. It would be of great benefit to these couples if an identifiable abnormality could be discovered to explain their problems not only in order to improve treatment options but also emotionally – a diagnosis is often easier to accept than a statistic. Ideally such an abnormality could be readily tested for, clearly identified by the appropriate test and correctable by simple means.

It is likely however, that the condition of unexplained subfertility is heterogeneous. Potential problems include oocyte abnormalities, dysfunction of the egg release mechanism or its follicular support, abnormalities of gamete transfer in the Fallopian tube, the chemical milieu of the tube itself, sperm abnormalities, abnormalities of the fertilisation process, abnormalities of the uterine environment, abnormalities of

embryo/endometrial interactions and finally abnormalities of the endometrium itself. In addition there will be other physical, psychological and social factors that may play a role in relative subfertility. Some of these problems may become apparent during the course of treatment; for example, normal appearing sperm may fail to fertilise normal appearing oocytes *in vitro*. This difficulty may be by-passed by the process of intra-cytoplasmic sperm injection (ICSI) and potentially improved by the various innovative treatments to the zona pellucida such as drilling (Fishel and Timson 1992). Sperm abnormalities may also be identified at IVF and corrected by ICSI. Tubal and endometrial environmental abnormalities could potentially be sampled via local cannulation but may be relatively labile and affected by the sampling process. Examination of the egg release mechanisms and embryo/endometrial interactions potentially disrupt a conception cycle. Endometrium is readily sampled in cycles where conception is avoided. There has been as yet, little clear evidence of morphological or functional abnormalities identifiable in the endometrium in relation to infertility however. The intimacy between the ovarian endocrine and endometrial cycles means that closer examination may yield cycle or hormone dependent abnormalities which may then be correctable.

Noyes et al. (1950) described the first useful examination of the cyclical changes occurring in endometrium. Subsequent workers have attempted to improve on this, recognising that more critical timing of biopsies, such as from ovulation rather than last or next menstrual period, as well as more precise examination, gave more reproducible results (Koninckx et al. 1977, Tredway et al. 1979, Johannisson et al. 1982, 1987, Shoupe et al. 1989, Li et al. 1987, 1988, 1989). The diagnosis of luteal

phase deficiency, still made by some empirically, has remained controversial during this debate (Li and Cooke 1991). It is difficult to consider so-called out of phase endometrium if there is no accurate anchor from which to date a specimen. In keeping with this lack of support for the diagnosis, which was considered in many cases to be the result of progesterone deficiency, there is no clear evidence of improved fertility following treatment with progestagens (Li and Cooke 1991). With the use of better dating techniques it is now considered likely that there is a cohort of women where the endometrium does not match the stage of cycle, not only at the histological level but also functionally, and in whom fertility may be abnormal (Lessey et al. 1992). If it is a cycle dependent abnormality it is to be hoped that correction may be possible by means of cycle manipulation, perhaps hormonally. There may also, however, be more static abnormalities which may again be detected, though perhaps less readily treated.

In this thesis various factors influential to the endometrium and in particular to its immunology have been considered and their study extended into the subfertile endometrium. The uterus has always been considered to be a privileged immunological site where embryos, composed of genetic material derived from both maternal and paternal DNA can thrive. Although in the early stages of development gene products are exclusively oocyte derived, around the time of implantation embryonic genes begin to be expressed (Watson 1992) such that there is the potential for maternal rejection of antigenically foreign material. Endometrial leucocyte populations have previously been well characterised and it is of note that whereas classical IgA-based mucosal immune systems have been detected in both cervix and

Fallopian tube, no such system occurs in the endometrium (Johnson et al. 1999). In fact the presence of plasma cells in the endometrium is indicative of endometritis (Loke and King 1995). The tolerance, which develops in the uterus to allow not only the survival of such foreign material but also its growth, is unique to this site and must be the most fundamental function of the endometrium.

It was considered valuable therefore, to consider the immunology of the endometrium in the first instance. Leucocyte populations of normal endometrium have been defined throughout the menstrual cycle (Bulmer et al. 1991, Starkey et al. 1991). The CD56 positive endometrial granulated lymphocytes, because of their dynamic population, have given rise to most interest. They are known to express some antigens characteristic of NK cells (Bulmer 1991, King and Loke 1991) and some NK cell activity and other functions have been identified *in vitro* (King and Loke 1990, Ferry et al. 1991). Their numbers increase in the late secretory phase of the menstrual cycle and this is at least in part, due to local proliferation (Pace et al. 1989, Tabibzadeh 1990).

The mechanisms that control the proliferation of eGLs have not been established. As it is cycle dependent it seems likely that steroid sex hormones may play a role either directly or indirectly. In this study the possibility of a direct influence of oestrogen and progesterone on any of the endometrial leucocyte populations has been excluded (Chapter 4). There was no evidence of expression of oestrogen or progesterone receptors by any of the relevant populations of cells. This does not in itself exclude the possibility of an indirect steroid influence, but makes a direct effect unlikely.

A second aspect of the control of eGL numbers is the question of apoptosis in the secretory phase. eGLs have an unusual morphology at this stage in the cycle which has given rise to the hypothesis that they are undergoing apoptosis as part of the termination of the secretory phase and onset of menses. High expression of bcl-2 antigen has in fact been reported in secretory phase endometrium which by virtue of its protective properties against apoptosis could negate this argument for programmed cell death. It is demonstrated in this study however, that only a small proportion of CD56 positive eGLs express bcl-2 such that the majority of the high level of bcl-2 expression in the late luteal phase exists in non-leucocyte stromal cells. The possibility of apoptosis has not been completely excluded by this study but additional evidence comes from Jones et al. (1998) where utilisation of TUNEL method of detection failed to confirm significant amounts of apoptosis in late luteal phase endometrium. This is also in keeping with work by Jones et al. (1997) showing that eGLs purified from pre-menstrual endometrium are functional.

Steroid hormones clearly have a fundamental role to play in the cyclicity of the endometrium but as they do not exert a direct influence on endometrial leucocytes it was of importance to discover whether an indirect steroid hormone effect may have a bearing on fertility. Oestrogen and progesterone receptor distribution in normal endometrium has been fully described previously. In this study the distribution in subfertile endometrium was also examined. Previous parity was also taken into account. It was of particular interest to find that there was lower endometrial surface epithelial progesterone receptor expression at LH+7, the time of implantation, in subfertile endometrium, a finding which was significant in the parous individuals. In

addition in the parous fertile subjects there was a significant reduction in endometrial surface epithelial progesterone receptor expression from LH+7 to LH+13 which was not seen in other groups. Thus for the first time a potential difference in endometrial control resulting from previous parity has been identified.

Only the study by Klentzeris et al. (1994) has suggested a difference in endometrial stromal leucocyte populations between fertile and subfertile endometrium. Following on from the steroid receptor studies quantification of endometrial leucocyte populations taking both parity and fertility status into account, was done. Although there were no significant differences numerically in the endometrial leucocyte populations in relation to parity or fertility, the population expansion of leucocytes did appear to differ in subfertile tissue. The increase in leucocyte numbers from LH+7 to LH+13 of which eGLs are primarily responsible is greater in fertile compared with subfertile endometrium. Although the function of eGLs has not been fully elucidated, it is likely that although they may not have direct impact on trophoblast invasion, by virtue of secreted cytokines and co-operation in endometrial messenger and immunological functioning, such differences may have a bearing on fertility itself.

This study has not confirmed the findings of Klentzeris et al. (1994) with regard to endometrial T cell sub-populations. Furthermore, examination of leucocyte activation molecules, although confirming previous reports, did not show altered status in subfertile tissue.

The work of Lessey et al. (1992, 1994, 1995) on adhesion molecules is well established and the idea of the window of implantation in relation to $\alpha_1\beta_1$, $\alpha_v\beta_3$ and $\alpha_4\beta_1$ is generally accepted. They have reported disordered expression of some adhesion molecules in the endometrium of the subfertile population resulting in the failure to produce the appropriate window for implantation. Reported here (Chapter 6) is the expression of a battery of relevant adhesion molecules in the endometrium of the four groups of subjects previously described. The general distribution and stage specific changes of these adhesion molecules were confirmed but no significant differences resulting from previous parity or fertility status were distinguished.

Although there have been no clear-cut abnormalities with regard to either endometrial leucocyte populations or their distant (steroid hormones) and local (adhesion molecules) controls there is a suggestion that endometrium of women with unexplained subfertility may differ to some extent from that of fertile controls. Further important work in this field would include an examination of the function of the lymphocytes themselves and the response of the endometrium, including the leucocyte populations to the embryo. Although eGLs have previously been examined for function, endometrial T cells in particular, have not. In the same way as eGLs differ from their peripheral blood counterparts it is unreasonable to assume that endometrial T cells are functionally equivalent to circulating T cells of which there are many sub-types. Consequently if the function of these cells is to be studied *in vitro* they need to be first isolated from endometrium. To this end a pilot study has been performed confirming that small populations of cells can be extracted from Pipelle biopsies of endometrium in sufficient numbers to perform relevant functional

assays. This has been performed successfully for the first time using the Mini MACS system and opens the way to closer examination of these cells form different groups of women and at different specified times of the cycle.

The examination of the embryo/endometrial interactions which are unique to the human species is not straightforward. Investigation of this function in humans is limited by legal and ethical considerations. The areas of human embryology and implantation *in vivo* are covered from a legal point of view by the 1967 Abortion Act and the 1990 Human Fertilisation and Embryology Act. There is some scope for licensed work on human embryos *in vitro* under the auspices of the Human Fertilisation and Embryology Authority (HFEA) but this is further limited by the availability of suitable material.

A second pilot study was performed to examine the possibility of testing the effect of embryo gene products on leucocyte functional assays. By using the small volumes afforded by the hanging drop technique of the Terasaki plates the use of individual embryo supernatants has been demonstrated. It is clear from the results that, certainly at blastocyst stage and in embryos of an equivalent age, there is a heterogeneity of effects of the supernatants on the standard mixed lymphocyte proliferation response and this brings into doubt the value of examining pooled supernatants which has only been reported before. Although Seifer et al. (1993) failed to demonstrate key cytokines, IL-1α, IL-1β, IL-6 and LIF using commercially available ELISA kits, individual supernatants could potentially be examined at a

variety of embryo stages, including those of transfer grade embryos at the time of embryo replacement thus allowing some information regarding their outcome to be obtained.

Although a definitive diagnostic test has not been elaborated from the work described in this thesis, it has been possible to confirm important features of the cyclical changes occurring in the endometrium at an immunohistochemical level and in particular to consider differences in the endometrium as a result of parity. Previously reported findings with regard to the endometrium in unexplained subfertility have been challenged and some confirmed. In addition useful pilot studies have been developed to allow more ready examination of other features of endometrial immunology and its control. Future work would aim to look more closely at the findings discussed in this thesis, in particular a closer examination of the steroid responsiveness of the endometrial superficial epithelium which is the initial site of contact with the embryo would be of interest in relation to previous parity. Perhaps the altered receptor status seen here has a role in receptivity for the establishment of subsequent pregnancies – it is generally accepted that parous subfertile women are easier to treat than nulliparous. A detailed study of isolated endometrial leucocytes would be of interest since their functions have not been fully established. In particular T cell populations which have now been shown to be amenable to isolation without cloning require further characterisation in the face of fertility issues. It would be of value to explore further the use of embryo culture supernatants in the investigation of endometrial/embryo interactions since the legislation surrounding the use of human embryos and the advances in technology

(freezing, *in vitro* maturation processes and changes in ovarian stimulation regimes) are likely to make these a scarcer resource for direct study in the future.

REFERENCES

REFERENCES

- Abelda, S.M. and Buck, C.A. (1990) Integrins and other cell adhesion molecules. *FASEB J.,* **4,** 2868-2880.
- Amso, N.N., Crow, J. and Shaw, R.W. (1994) Comparative immunohistochemical study of oestrogen and progesterone receptors in the Fallopian tube and uterus at different stages of the menstrual cycle and the menopause. *Hum. Reprod.* **9,** 1027-1037.
- Anderson, R.A., Eccles, S.E. and Irvine, D.S. (1996) Home ovulation testing in a donor insemination service. *Hum. Reprod.,* **11,** 1674-1677.
- Andrew, S.M. and Jasani, B. (1987) An improved method for the inhibition of endogenous peroxidase non-deleterious to lymphocyte surface markers. Application to immunoperoxidase studies on eosinophil-rich tissue preparations. *Histochem. J.,* **19,** 426-430.
- Aplin, J.D., Charlton, A.K. and Ayad, S. (1988) An immunohistochemical study of human endometrial extracellular matrix during the menstrual cycle and first trimester of pregnancy. *Cell Tissue Res.,* **253,** 231-240.
- Arici, A., Engin, O., Attar, E. and Olive, D.L. (1995) Modulation of leukaemia inhibitory factor gene expression and protein biosynthesis in human endometrium. *J. Clin. Endocrinol. Metab.,* **80,** 1908-1915.
- Athanassakis, I., Bleackley, R.C., Paetkau, V., Guilbert, L., Barr, P.J. and Wegmann, T.G. (1987) The immunostimulatory effect of T cells and T cell lymphokines on murine fetally derived placental cells. *J. Immunol.,* **138,** 37-44.

- Balasch, J., Creus, M., Marquez, M., Burzaco, I. and Vanrell, J.A. (1986) The significance of luteal phase deficiency on fertility: A diagnostic and therapeutic approach. *Hum. Reprod.,* **1,** 145-147.
- Balasch, J., Fábregues, F., Creus, M. and Vanrell, J.A. (1992) The usefulness of endometrial biopsy for luteal phase evaluation in infertility. *Hum. Reprod.,* **7,** 973-977.
- Bednarczyk, J.L. and McIntyre, B.W. (1990) A monoclonal antibody to VLA-4 α-chain (CDW49d) induces homotypic lymphocyte aggregation. *J. Immunol.,* **44,** 777-784.
- Bergh, P.A. and Navot, D. (1992) The impact of embryonic development and endometrial maturity on the timing of implantation. *Fertil. Steril.,* **58,** 537-542.
- Bilalis, D.A., Klentzeris, L.D. and Fleming, S. (1996) Immunohistochemical localization of extracellular matrix proteins in luteal phase endometrium of fertile and infertile patients. *Hum. Reprod.,* **11,** 2713-2718.
- Bischof, P., Haenggeli, L. and Campana, A. (1995) Effect of leukaemia inhibitory factor on human cytotrophoblast differentiation along the invasive pathway. *Am. J. Reprod. Immunol.,* **34,** 225-230.
- Brown, R. (1996) The bcl-2 family of proteins. *Br. Med. J.,* **53,** 466-477.
- Bulmer, J.N. and Sunderland, C.A. (1984) Immunohistological characterization of lymphoid cell populations in the early human placental bed. *Immunol.,* **52,** 349-357.
- Bulmer, J.N., Hollinger, D. and Ritson, A. (1987) Immunocytochemical evidence that endometrial stromal granulocytes are granulated lymphocytes. *J. Pathol.,* **153,** 281-288.

- Bulmer, J.N., Longfellow, M. and Ritson, A. (1991a) Leukocytes and resident blood cells in endometrium. *Ann. N. Y. Acad. Sci.,* **622,** 57-68.
- Bulmer, J.N., Morrison, L., Longfellow, M., Ritson, A. and Pace, D. (1991b) Granulated lymphocytes in human endometrium: histochemical and immunohistochemical studies. *Hum. Reprod.,* **6,** 791-798.
- Cattoretti, G., Pileri, S., Parravicini, C., Becker, H.G., Poggi, S., Bifulco, C., Key, G., D'Amato, L., Sabattini, E., Feudale, E., Reynolds, F., Gerdes, J. and Rilke, F. (1993) Antigen unmasking on formalin-fixed, paraffin-embedded tissue sections. *J. Path.,* **171,** 83-98.
- Charnock-Jones, D.S., Sharkey, A.M., Fenwick, P. and Smith, S.K. (1994) Leukaemia inhibitory factor mRNA concentration peaks in human endometrium at the time of implantation and the blastocyst contains mRNA for the receptor at this time. *J. Repro. Fertil.,* **101,** 421-426.
- Chauchereau, A., Savouret, J-F. and Milgram, E. (1992) Control of biosynthesis and post transcriptional modification of progesterone receptors. *Biol. Reprod.,* **46,** 174-177.
- Chen, C-K., Huang, S-C., Chen, C-L., Yen, M-R., Hsu, H-C. and Ho, H-N. (1995) Increased expression of CD69 and HLA-DR but not of CD25 or CD71 on endometrial T lymphocytes of non-pregnant women. *Hum. Immunol.,* **42,** 227-232.
- Christmas, S.E., Bulmer, J.N., Meager, A. and Johnson, P.M. (1990) Phenotypic and functional analysis of human $CD3^-$ decidual leucocyte clones. *Immunol.,* **71,** 182-189.

- Christmas, S.E. and Johnson P.M. (1996) Fundamental Immunology In: Hillier, S.G., Kitchener, H.C. and Neilson, J.P. (Eds.). *Scientific essentials: reproductive medicine.* W.B. Saunders, UK. 60-71.
- Clark, D.A., Vima, G., Flanders, K.C., Hirte, H. and Starkey, P. (1994) $CD56^{+}$ lymphoid cells in human first trimester pregnancy decidua as a source of novel transforming growth factor-β_2-related immunosuppressive factors. *Hum. Reprod.,* **9,** 2270-2277.
- Coppens, M.T., Dhont, M.A., De Boever, J.G., Sereyn, R.F., Vandeherckhove, D.A. and Raels, H.J. (1993) The distribution of oestrogen and progesterone receptors in the human endometrial basal and functional layer during the normal menstrual cycle. *Histochem.,* **99,** 121-126.
- Corsan, G.H., Ghazi, D. and Kemmann, E. (1990) Home urinary luteinizing hormone immuno assays: clinical applications. *Fertil. Steril.,* **53,** 591-601.
- Critchley, H.O.D., Bailey, D.A., Au, C.L., Affandi, B. and Rogers, P.A.W. (1993) Immunohistochemical sex steroid receptor distribution in endometrium of long-term subdermal levonorgestrel users and during the normal menstrual cycle. *Hum. Reprod.,* **8,** 1632-1639.
- Critchley, H.O.D. (1996) Uterus and tubes. In: Hillier, S.G., Kitchener, H.C. and Neilson, J.P. (Eds.). *Scientific essentials: reproductive medicine.* W.B. Saunders, UK. 184-195.
- Critchley, H.O.D., Kelly, R.W., Lea, R.G., Drudy, T.A., Jones, R.L. and Baird, D.T. (1996) Sex steroid regulation of leukocyte traffic in human decidua. *Hum. Reprod.,* **11,** 2257-2262.

- Croy, B.A., Ashkar, A.A., Foster, R.A., DiSanto, J.P., Magram, J., Carson, D., Gendler, S.J., Grusby, M.J., Wagner, N. and Muller, W. (1997) Histological studies of gene-ablated mice support important functional roles for natural killer cells in the uterus during pregnancy. *J. Reprod. Immunol.*, **35,** 111-133.
- Cullinan, E.B., Abbondanzo, S.J., Anderson, P.S., Pollard, J.W., Lessey, B.A. and Stewart, C.L. (1996) Leukaemia inhibitory factor (LIF) and LIF receptor expression in human endometrium suggests a potential autocrine / paracrine function in regulating embryo implantation. *Proc. Natl. Acad. Sci. USA,* **93,** 3115-3120.
- Dallenbach-Hellweg, G. (1981) The normal histology of the endometrium. In: Dallenbach-Hellweg, G. (Ed.). *Histopathology of the endometrium. 3rd Edn.* Springer-Verlag, Berlin. 22-88.
- Daly, D.C., Walters, C.A., Soto-Albors, C.E. and Riddick, D.H. (1983) Endometrial biopsy during treatment of luteal phase defects is predictive of therapeutic outcome. *Fertil. Steril.,* **40,** 305-310.
- Damsky, C.H., Librach, C., Lim, K.-H., Fitzgerald, M.L., McMaster, M.T., Janatpour, M., Zhou, Y., Logan, S.K., and Fisher, S.J. (1994) Integrin switching regulates normal trophoblast invasion. *Development,* **120,** 3657-3666.
- Das, S.K., Wang, X.-N., Paria, B.C., Damm, D., Abraham, J.A., Klagsbrun, M., Andrews G.K. and Dey, S.K. (1994) Heparin-binding EGF-like growth factor gene is induced in the mouse uterus temporally by the blastocyst solely at the site of its apposition: a possible ligand for interaction with blastocyst EGF-receptor in implantation. *Development,* **120,** 1071-1083.

- Davidson, B.J., Thrasher, T.V. and Seraj, I.M. (1987) An analysis of endometrial biopsies performed for infertility. *Fertil. Steril.,* **48,** 770-774.
- Deniz, G., Christmas, S.E., Brew, R. and Johnson, P.M. (1994) Phenotypic and functional cellular differences between human CD3- decidual and peripheral blood leukocytes. *J. Immunol.,* **152,** 4255-4261.
- Ekert, P.G. and Vaux, D.L. (1997) Apoptosis and the immune system. *Br. Med. Bull.,* **53,** 591-603.
- Ellis, S.A., Strachan, T., Palmer, M.S. and McMichael, A.J. (1989) Complete nucleotide sequence of a unique HLA class 1C locus product expressed on the human choriocarcinoma cell line BeWo. *J. Immunol.,* **142,** 3281-3285.
- Ellis, S.A., Palmer, M.S. and McMichael, A.J. (1990) Human trophoblast and the choriocarcinoma cell line BeWo express a truncated HLA class I molecule. *J. Immunol.,* **144,** 731-735.
- Ferenczy, A., Richard, R.M., Agate, F.J.Jr., Purkenson, M.L. and Dempsey, E.W. (1972) Scanning electron microscopy of the human endometrium surface epithelium. *Fertil. Steril.,* **23,** 515-521.
- Ferry, B.L., Sargent, I.L., Starkey, P.M. and Redman, C.W.G. (1991) Cytotoxic activity against trophoblast and choriocarcinoma cells of large granular lymphocytes from human early pregnancy decidua. *Cell. Immunol.,* **132,** 140-149.
- Fishel, S.B. and Timson, J. (1992) Sperm-egg manipulation for the treatment of male infertility. In: Templeton, A.A. and Drife, J.O. (Eds.) *Infertility,* Springer-Verlag, London. 115-132.

- Fleming. S. (1990) Cellular functions of adhesion molecules. *J. Path.,* **161,** 189-190.
- Garrod, D.R. (1993) Cell to cell and cell to matrix adhesion. *Br. Med. J.,* **306,** 703-705.
- Garcia, E., Bouchard, J., de Brux, J., Berdah, J., Frydman, R., Schaison, G., Milgrom, E. and Perrot-Applanat, M. (1988) Use of immunocytochemistry of progesterone and estrogen receptors for endometrial dating. *J. Clin. Endocrinol. Metab.,* **67,** 80-87.
- Giudice, L.C. (1999) Potential biochemical markers of uterine receptivity. *Hum. Reprod.,* **14 (Suppl.2),** 3-16.
- Guimond, M.J., Luross, J.A., Wang, B., Terhorst, C., Danial, S. and Croy, B.A. (1997) Absence of natural killer cells during murine pregnancy is associated with reproductive compromise in TgE26 mice. *Biol. Reprod.,* **56,** 169-179.
- Gold, R., Schmied, M., Giegerich, G., Breitschopf, H., Hartung, H.P., Toyka, K.V. and Lassmann, H. (1994) Differentiation between cellular apoptosis and necrosis by the combined use of in situ tailing and nick translation techniques. *Lab. Invest.,* **71,** 219-225.
- Gompel, A., Sabourin, J.C., Martin, A., Yaneva, H., Audouin, J., Decroix, Y. and Poitout, P. (1994) Bcl-2 expression in normal endometrium during the menstrual cycle. *Am. J. Pathol.,* **144,** 1195-1202.
- Good, R.G. and Moyer, D.L. (1968) Estrogen-progesterone relationship in the development of secretory endometrium. *Fertil. Steril.,* **19,** 37-49.

- Graham, R.A., Seif, M.W., Aplin, J.D., Li, T.C., Cooke, I.D., Rogers, A.W. and Dockery, P. (1990) An endometrial factor in unexplained infertility. *Br. Med. J.,* **300,** 1428-1431.
- Grosskinsky, C.M., Yowell, C.W., Sun, J., Parise, L.V. and Lessey, B.A. (1996) Modulation of integrin expression in endometrial stromal cells in vitro. *J. Clin. Endocrinol. Metab.,* **81,** 2047-2054.
- Hamperl, H. (1955) The granular endometrial stromal cells: a new cell type. *J. Pathol. Bacteriol.,* **69,** 358-359.
- Hamperl, H. and Hellweg, G. (1958) Granular endometrial stroma cells. *Obstet, Gynecol.,* **11,** 379-387.
- Hey, N.A., Graham, R.A., Seif, M.W. and Aplin, J.D. (1990) The polymorphic epithelial mucin MUC-1 in human endometrium is regulated with maximal expression in the implantation phase. *J. Clin. Endocrinol. Metab.,* **78,** 337-342.
- Heybourne, K., Fu, Y.-X., Nelson, A., Farr, A., O'Brien, R. and Born, W. (1994) Recognition of trophoblasts by γδ T cells. *J. Immunol.,* **153,** 2918-2916.
- Hsu, S-M., Raine, L. and Fanger, H. (1981) Use of Avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabelled antibody (PAP) procedures. *J. Histochem. Cytochem.,* **29,** 577-580.
- Hull, M.G., Glazener, C.M., Kelly, N.J., Conway, D.I., Foster, P.A., Hinton, R.A., Coulson, C., Lambert, P.A., Watt, E.M. and Desai, K.M. (1985) Population study of causes, treatment and outcome of infertility. *Br. Med. J. (Clin. Res. Ed.),* **291,** 1693-1697.

- Hull, M.G.R. (1992) The causes of infertility and relative effectiveness of treatment. In: Templeton, A.A. and Drife, J.O. (Eds.) *Infertility,* Springer-Verlag, London. 33-62.
- Human Fertilisation and Embryology Act (1990). HMSO.
- Hynes, R.O. (1987) Integrins a family of cell surface receptors. *Cell,* **48,** 549-555.
- Hynes, R.O. (1992) Integrins: Versatility, modulation and signalling in cell adhesion. *Cell,* **69,** 11-25.
- Hynes, R.O. (1996) Targeted mutations in cell adhesion genes: what have we learned from them? *Dev. Biol.,* **180,** 402-412.
- Inoue, T., Kanzaki, H., Imai, K., Narukawa, S. Katsuragawa, H., Watanabe, H., Hirano, T. and Mori, T. (1996) Progesterone stimulates the induction of human endometrial CD56+ lymphocytes in an in vitro culture system. *J. Clin. Endocrinol. Metab.,* **81,** 1502-1507.
- Jequier A.M. (1995) Clinical disorders affecting semen quality. In: Grudzinskas, J.G. and Yovich, J.L. (eds.) *Gametes. The spermatozoon.,* Cambridge University Press, UK. 175-191.
- Johannisson, E., Parker, R.A., Landgren, B-M. and Diczfalusy, E. (1982) Morphometric analysis of the human endometrium in relation to peripheral hormone levels. *Fertil. Steril.,* **38,** 564-571.
- Johannisson, E., Landgren, B-M., Rohr, H.P. and Diczfalusy, E. (1987) Endometrial morphology and peripheral hormone levels in women with regular menstrual cycles. *Fertil. Steril.,* **48,** 401-408.

- Johnson, P.M. and Christmas, S.E. (1996) Immunology in reproduction. In: Hillier, S.G., Kitchener, H.C. and Neilson, J.P. (Eds.). *Scientific essentials: reproductive medicine.* W.B. Saunders, UK. 284-291.
- Johnson, P.M., Christmas, S.E. and Vince, G.S. (1999) Immunological aspects of implantation and implantation failure. *Hum. Reprod.,* **14 (Suppl. 2),** 26-36.
- Jones, G.E.S. (1949) Some newer aspects of the management of infertility. *J.A.M.A.,* **14,** 1123-1129.
- Jones, S., Vince, G., Herrington, P. and Johnson, P.M. (1997a) Endometrial large granular lymphocytes: correlation with IVF outcome. *J. Reprod. Immunol.,* **34,** 54-55.
- Jones, R.L., Kelly, R.W. and Critchley, H.O.D. (1997b) Chemokine and cyclooxygenase-2 expression in human endometrium coincides with leukocyte accumulation. *Hum. Reprod.,* **12,** 1300-1306.
- Jones, R.K., Searle, R.F. and Bulmer, J.N. (1997c) Cytotoxic activity of endometrial granulated lymphocytes during the menstrual cycle in humans. *Biol. Reprod.,* **57,** 1217-1222.
- Jones, R.K., Searle, R.F., Stewart, J.A., Turner, S. and Bulmer, J.N. (1998a) Apoptosis, bcl-2 expression and proliferative activity in human endometrial stroma and endometrial granulated lymphocytes. *Biol. Reprod.,* **58,** 995-1002.
- Jones, R.K., Searle, R.F. and Bulmer, J.N. (1998b) Apoptosis and bcl-2 expression in normal human endometrium, endometriosis and adenomyosis. *Hum. Reprod.,* **13,** 3496-3502.

- Jordan, J., Craig, K., Clifton, D.K. and Soules, M.R. (1994) Luteal phase defect: The sensitivity and specificity of diagnostic methods in common use. *Fertil. Steril.,* **62,** 54-62.
- King, A. Wellings, V., Gardner, L. and Loke, Y.W. (1989a) Immunocytochemical characterisation of the unusual large granular lymphocytes in human endometrium throughout the menstrual cycle. *Hum. Immunol.,* **24,** 195-205.
- King, A., Birkby, C. and Loke, Y.W. (1989b) Early human decidual cells exhibit NK activity against the K562 cell line but not against first trimester trophoblast. *Cell. Immunol.,* **118,** 337-344.
- King, A. and Loke, Y.W. (1990a) Human trophoblast and JEG choriocarcinoma cells are sensitive to lysis by Il-2-stimulated decidual NK cells. *Cell. Immunol.,* **129,** 435-448.
- King, A. and Loke, Y.W. (1990b) Uterine large granular lymphocytes: a possible role in embryonic implantation? *Am. J. Obstet. Gynecol.,* **162,** 308-310.
- King, A. and Loke, Y.W. (1991) On the nature and function of human uterine granular lymphocytes. *Immunol. Today,* **12,** 432-435.
- King, A., Balendran, N., Wooding, P., Carter, N.P. and Loke, Y.W. (1991) CD3- leukocytes present in the human uterus during early placentation: phenotypic and morphologic characterisation of the CD56++ population. *Dev. Immunol.,* **1,** 169-190.
- King, A., Wooding, P., Gardner, L. and Loke, Y.W. (1993) Expression of perforin, granzyme A and TIA-1 by human uterine CD56+ NK cells implies they are activated and capable of effector functions. *Hum. Reprod.,* **8,** 2061-2067.

- King, A., Gardner, L. and Loke, Y.W. (1996) Evaluation of oestrogen and progesterone receptor expression in uterine mucosal lymphocytes. *Hum. Reprod.,* **11,** 1079-1082.
- Klentzeris, L.D., Bulmer, J.N., Warren, A., Morrison, L., Li, T-C. and Cooke, I.D. (1992) Endometrial lymphoid tissue in the timed endometrial biopsy: morphometric and immunohistochemical aspects. *Am. J. Obstet. Gynecol.,* **167,** 667-674.
- Klentzeris L.D., Bulmer, J.N., Trejdosiewicz, L.K., Morrison, L. and Cooke, I.D. (1993) Beta-1 integrin cell adhesion molecules in the endometrium of fertile and infertile women. *Hum. Reprod.,* **8,** 1223-1230.
- Klentzeris, L.D., Bulmer, J.N., Warren, M.A., Morrison, L., Li, T.C. and Cooke, I.D. (1994) Lymphoid tissue in the endometrium of women with unexplained infertility: morphometric and immunohistochemical aspects. *Hum. Reprod.,* **9,** 646-652.
- Kliman, H.J., Feinberg, R.F., Schwartz, L.B., Feinman, M.A., Lavi, E. and Meaddough, E.L. (1995) A mucin-like glycoprotein identified by MAG (mouse ascites golgi) antibodies. Menstrual cycle-dependent localization in human endometrium. *Am. J. Pathol.,* **146,** 166-181.
- Koh, E.A.T., Illingworth, P.J., Duncan, W.C. and Critchley, H.O.D. (1995) Immunolocalization of bcl-2 protein in human endometrium in the menstrual cycle and stimulated early pregnancy. *Mol. Hum. Reprod.,* **1,** 1557-1562.

- Kojima, K., Kanzaki, H., Iwai, M., Hatayama, H., Fujimoto, M., Inoue, T., Horie, K., Nakayama, H., Fujita, J. and Mori, T. (1994) Expression of leukaemia inhibitory factor in human endometrium and placenta. *Biol. Reprod.,* **50,** 882-887.
- Koninckx, P.R., Goddeeris, P.G., Lauweryns, J.M., Dettertogh, R.C. and Brosens, I.A. (1977) Accuracy of endometrial biopsy dating in relation to the midcycle luteinizing hormone peak. *Fertil. Steril.,* **28,** 443-445.
- Kos, F.J. and Engleman, E.G. (1995) Requirement for natural killer cells in the induction of cytotoxic T cells. *J. Immunol.,* **155,** 578-584.
- Kovats, S., Main, E.K., Librach, C., Stubblebine, M., Fisher, S.J. and De Mars, R. (1990) A class I antigens, HLA-G, expressed in human trophoblasts. *Science,* **248,** 220-223.
- Lenton, E.A., Landgren, B-M. and Sexton, L. (1984) Normal variation in the length of the luteal phase of the menstrual cycle: identification of the short luteal phase. *Br. J. Obstet. Gynaecol.,* **91,** 685-689.
- Lessey, B.A., Killam, A.P., Metzger, D.A., Haney, A.F., Greene, G.L. and McCarty Jr., K.S. (1988) Immunohistochemical analysis of human uterine estrogen and progesterone receptors throughout the menstrual cycle. *J. Clin. Endocrinol. Metab.,* **67,** 334-340.
- Lessey, B.A., Damjanovich, L., Coutifaris, C., Castelbaum, A., Albelda, S.M. and Buck, C.A. (1992) Integrin adhesion molecules in human endometrium: correlation with the normal and abnormal menstrual cycle. *J. Clin. Invest.,* **90,** 188-195.

- Lessey, B.A., Castelbaum, A.J., Buck, C.A., Lei, Y., Yowell, C.W. and Sun, J. (1994) Further characterisation of endometrial integrins during the menstrual cycle and in pregnancy. *Fertil. Steril.*, **62,** 497-506.
- Lessey, B.A. (1995) Integrins as markers of uterine receptivity in women with primary unexplained infertility. *Fertil. Steril.*, **63,** 535-542.
- Lessey, B.A., Ilesanmi, A.O., Lessey, M.A., Riben, M., Harris, J.E. and Chwalisz, K. (1996) Luminal and glandular endometrial epithelia express integrins differentially throughout the menstrual cycle: implications for implantation, contraception and infertility. *Am. J. Reprod, Immunol.*, **35,** 195-204.
- Lessey, B.A., Yeh, I., Castelbaum, A.J., Fritz, M.A., Ilesamni, A.O., Korzeniowshi, A., Sun, J. and Chwalisz, K. (1996) Endometrial progesterone receptors and markers of uterine receptivity in the window of implantation. *Fertil. Steril.*, **65,** 477-483.
- Levy, C., Robel, P., Gautray, J.P., de Brux, J., Verma, U., Descomps, B., Baulieu, E.E. and Eychenne, B. (1980) Estradiol and progesterone receptors in human endometrium: normal and abnormal menstrual cycles and early pregnancy. *Am. J. Obstet. Gynecol.*, **136,** 646-651.
- Li, T-C., Rogers, A.W., Lenton, E.A., Dockery, P. and Cooke, I. (1987) A comparison between two methods of chronological dating of human endometrial biopsies during the luteal phase and their correlation with histological dating. *Fertil. Steril.*, **48,** 928-932.

- Li, T-C., Rogers, A.W., Dockery, P., Lenton, E.A. and Cooke, I.D. (1988) A new method of histologic dating of human endometrium in the luteal phase. *Fertil. Steril.*, **50,** 52-60.
- Li, T-C., Dockery, P., Rogers, A.W. and Cooke, I.D. (1989a) How precise is histologic dating of endometrium using the standard dating criteria? *Fertil. Steril.*, **51,** 759-763.
- Li, T.C., Lenton, E.A., Dockery, P., Rogers, A.W. and Cooke, I.D. (1989b) The relatio between daily salivary progesterone profile and endometrial development in the luteal phase of fertile and infertile women. *Br. J. Obstet. Gynaecol.*, **96,** 445-453.
- Li, T-C. and Cooke, I.D. (1990) Outpatient endometrial biopsy: clinical, endocrinologic and histologic consequences. *Int. J. Gynecol. Obstet.*, **31,** 35-41.
- Li, T.C., Lenton, E.A., Dockery, P. and Cooke, I.D. (1990) A comparison of some clinical and endocrinological features between cycles with normal and defective luteal phases in women with unexplained infertility. *Hum. Reprod.*, **5,** 805-810.
- Li, T.C. and Cooke, I.D. (1991) Evaluation of the luteal phase. *Hum. Reprod.*, **6,** 484-499.
- Lin, H., Mosmann, T.R., Guilbert, L., Tuntipopipat, S. and Wegman, T.G. (1993) Synthesis of T helper-2-type cytokines at the maternal fetal interface. *J. Immunol.*, **151,** 4562-4573.
- Liu, C-C., Parr, E.L. and Young, J.D. (1994) Granulated lymphoid cells of the pregnant uterus: morphological and functional features. *Int. Rev. Cytol.*, **153,** 105-136.

- Ljunggren, H-G and Kärre, K. (1990) In search of the "missing self": MHC molecules and natural killer cell recognition. *Immunol. Today,* **11,** 237-244.
- Loke, Y.W. and King, A. (1995) Human Implantation. Cambridge University Press, Cambridge.
- Manaseki, S. and Searle, R.F. (1989) Natural Killer (NK) cell activity of first trimester human decidua. *Cell. Immunol.,* **121,** 166-173.
- March CM. (1991) The endometrium in the menstrual cycle. In: Mishell DR, Davajan V, Lobo RA. (Eds.*). Infertility, contraception and reproductive endocrinology (3rd edition).* Blackwell Scientific, Boston, USA. 125-139.
- Martel, D., Monier, M.N., Roche, D. and Psychoyos, A. (1991) Hormonal dependence of pinopode formation at the uterine luminal surface. *Hum. Reprod.,* **6,** 597-603.
- Marzusch, K., Ruck, P., Gewelhart, A., Haudgetringer, R., Dietl, J.A., Kaiserling, E., Horny, H.-P., Vince, G. and Redman, C.W.G. (1993) Distribution of cell adhesion molecules on CD56++, CD3-, CD16- large granular lymphocytes and endothelial cells in first trimester human decidua. *Hum. Reprod.,* **8,** 1203-1208.
- Monterroso, V.H. and Hansen, P.J. (1993) Regulation of bovine and ovine lymphocyte proliferation: modulation by steroid receptor antagonists and physiological status. *Acta Endocrinologica,* **129,** 532-535.
- Murray, M.J, Zhang, J. and Lessey, B.A. (1999) Expression of α_6 and β_4 integrin subunitsthroughout the menstrual cycle: no correlation with uterine receptivity. *Fertil. Steril.,* **72,** 522-526.

- Nachtigall, M.J., Kliman, H.J., Feinberg, R.F., Olive, D.L., Engin, O. and Arici, A. (1996) The effect of leukaemia inhibitory factor (LIF) on trophoblast differentiation: a potential role in human implantation. *J. Clin. Endocrinol. Metab.*, **81,** 801 806.
- Neifeld, J.P., Lippman, M.E. and Tormey, D.C. (1977) Steroid hormone receptors in normal human lymphocytes. *J. Biol. Chem.*, **252,** 2972-2977.
- Nieder, G.L., Weitlauf, H.M. and Suda-Hartman, M. (1987) Synthesis and secretion of stage specific proteins by peri-implantation mouse embryos. *Biol. Reprod.*, **36,** 687-699.
- Norton, A.J. (1993) Microwave oven heating for antigen unmasking in routinely processed tissue sections. *J. Pathol.*, **171,** 79-80.
- Noyes, R.W., Hertig, A.T. and Rock, J. (1950) Dating the endometrial biopsy. *Fertil. Steril.*, **1,** 3-25.
- Otsuki, Y. Misaki, O., Sugimoto, O., Ito, Y., Tsujimoto, Y. and Akao, Y. (1994) Cyclic bcl-2 gene expression in human uterine endometrium during menstrual cycle. *Lancet,* **344,** 28-29.
- Pace, D., Morrison, L. and Bulmer, J.N. (1989) Proliferative activity in endometrial stromal granulocytes throughout menstrual cycle and normal pregnancy. *J. Clin. Pathol.*, **42,** 35-39.
- Press, M.F., Nousek-Goebl, N.A., Bur, M. and Greene, G.L. (1986) Estrogen receptor localization in the female genital tract. *Am. J. Pathol.*, **123,** 280-292.
- Press, M.F., Udove, J.A. and Greene, G.L. (1988) Progesterone distribution in the human endometrium. *Am. J. Pathol.*, **131,** 112-124.

- Rasheed, F.N., Bulmer, J.N., Dunn, D.T., Menendez, C., Jawa, M.F.B., Jepson, A., Jakobsen, P.H. and Greenwood, B.M. (1993) Suppressed peripheral and placental blood lympho-proliferative responses in first pregnancies: relevance to malaria. *Am. J. Trop. Med. Hyg.*, **48,** 154-160.
- RCOG (1992) Infertility: Guidelines for practice. RCOG Press.
- RCOG (1998) The initial investigation and management of the infertile couple. In: *Evidence-based clinical guidelines*. **2,** 56-59.
- Reed, J.C. (1994) bcl-2 and the regulation of programmed cell death. *J. Cell Biol.*, **124,** 1-6.
- Ritson, A. and Bulmer, J.N. (1987) Endometrial granulocytes in human decidua react with natural killer (NK) cell marker NKH1. *Immunology,* **62,** 329-331.
- Ritson, A. and Bulmer, J.N., (1989) Isolation and functional studies of granulated lymphocytes in first trimester human decidua. *Clin. Exp. Immunol.*, **77,** 263-268.
- Robertson, S.A., Mau, V.J., Hudson, S.N. and Tremellen, K.P. (1997) Cytokine-leucocyte networks and the establishment of pregnancy. *Am. J. Reprod. Immunol.*, **37,** 438-442.
- Roitt, I.M. (1994) Essential Immunology. Blackwell Scientific Publications. Oxford.
- Rosenberg, S.M., Luciano, A.A. and Riddick, D.H. (1980) The luteal phase defect: the relative frequency of, and encouraging response to treatment with vaginal progesterone. *Fertil. Steril.*, **34,** 17-20.
- Saito, S., Nishikawa, K., Morii, T., Enomoto, M., Narita, N., Motoyoshi, K. and Ichijo, M. (1993) Cytokine production by $CD16^{-}$ $CD56^{bright}$ natural killer cells in the human early pregnancy decidua. *Int. Immunol.*, **5,** 559-563.

- Saito, S., Umekage, H., Nishikawa, K., Morii, T., Narita, N., Enomoto, M., Sakakura, S., Horad, N., Ichijo, M. and Marikawa, H. (1996) Interleukin 4 (IL-4) blocks the IL-2 induced increase in natural killer activity and DNA synthesis of decidual $CD16^{-}$ $CD56^{bright}$ NK cells by inhibiting expression of the IL-2 receptor α, β and γ. *Cell. Immunol.,* **170,** 71-77.
- Schmutzler, R.K., Diedrich, K. and Krebs, D. (1995) [Value of the Clearplan ovulation test in sterility treatment]. [German]. *Geburtshilte und Frauenheilkunde,* **55,** 266-269.
- Seifer, D.B., Romero, R., Berlinsky, D. and Haning Jr., R.V. (1993) Absence of immunoreactive cytokines in supernatants of individual preimplantation human embryos. *Am. J. Reprod. Immunol.,* **30,** 105-107.
- Shoupe, D., Mishell Jr., D.R., Lacarra, M., Lobo Roger, A., Horenstein, J., d'Ablaing, G. and Moyer, D. (1989) Correlation of endometrial maturation with four methods of estimating day of ovulation. *Obstet. Gynaecol.,* **73,** 88-92.
- Simón, C., Piquette, G., Frances, A. and Polan, M.L. (1993) Localisation of interleukin-1 type 1 receptor and interleukin-1β in human endometrium throughout the endometrial cycle. *J. Clin. Endocrinol. Metab.,* **77,** 549-555.
- Simón, C., Frances, A., Piquette, G., Hendrickson, M., Milki, A. and Polan, M.L. (1994) Interleukin-1 system in the materno-trophoblast unit in human implantation: immunohistochemical evidence for autocrine / paracrine function. *J. Clin. Endocrinol. Metab.,* **78,** 847-854.
- Simón, C. and Pellicer, A. (1995) Regulators of human implantation. *Hum. Reprod.,* **10 (Suppl. 2).** Oxford University Press. UK.

- Simón, C., Gineno, M.J., Mercador, A., O'Connor, J.E., Remokí, J., Polan, M.L. and Pellicer, A. (1997) Embryonic regulation of integrins β_3, α_4 and α_1 in human endometrial epithelial cells in vitro. *J. Clin. Endocrinol. Metab.*, **82,** 2607-2616.
- Simón, C., Moreno, C., Remohí, J. and Pellicer, A. (1998) Molecular interactions between embryo and uterus in the adhesion phase of human implantation. *Hum. Reprod.*, **13 (Suppl. 3),** 219-232.
- Simón, C., Pellicer, A. and Remohí, J. (1999) Emerging concepts on human implantation. *Hum. Reprod.*, **14 (Suppl. 2).** Oxford University Press. UK.
- Smith, S.K., Lenton, E.A., Landgren, B-M. and Cooke, I.D. (1984) The short luteal phase and infertility. *Br. J. Obstet. Gynaecol.*, **91,** 1120-1122.
- Snijders, M.P.M.L., de Goeij, A.F.P.M., Debets-Te Baerts, M.J.C., Rousch, M.J.M., Koudstaal, J. and Bosman F.T. (1992) Immunocytochemical analysis of oestrogen receptors and progesterone receptors in the human uterus throughout the menstrual cycle and after the menopause. *J. Reprod. Fertil.*, **94,** 363-371.
- Soules, M.R., Wiebe, R.H., Aksel, S. and Hammond, C.B. (1997) The diagnosis and therapy of luteal phase deficiency. *Fertil. Steril.*, **28,** 1033-1037.
- Starkey, P.M., Clover, L.M. and Rees, M.C.P. (1991) Variation during the menstrual cycle of immune cell populations in human endometrium. *Eur. J. Obstet. Gynecol. Reprod. Biol.*, **39,** 203-207.
- Starkey, P.M. (1991) Expression on cells of early human pregnancy decidua of the p75, IL-2 and p145, IL-4 receptor proteins. *Immunol.*, **73,** 64-70.

- Stewart, C.L., Kaspar, P., Brunet, L.J., Bhatt, H., Gadil, I., Kontgen, F. and Abbondanzo, S.J. (1992) Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. *Nature,* **359,** 76-79.
- Szekeres-Bartho, J., Szekeres, G., Debre, P., Autran, B. and Chaouat, G. (1990) Reactivity of lymphocytes to a progesterone receptor-specific monoclonal antibody. *Cell. Immunol.,* **125,** 273-283.
- Tabibzadeh, S.S. and Satyaswaroop, P.G. (1989) Sex steroid receptors in lymphoid cells of human endometrium. *Am. J. Clin. Pathol.,* **91,** 656-663.
- Tabibzadeh, S. (1990a) Proliferative activity of lymphoid cells in human endometrium throughout the menstrual cycle. *J. Clin. Endocrinol. Metab.,* **70,** 437-443.
- Tabibzadeh, S.S. (1990b) Evidence of T cell activation and potential cytokine action in human endometrium. *J. Endocrinol. Metab.,* **74,** 437-443.
- Tabibzadeh, S.S. and Poubouridis, D. (1990) Expression of Leukocyte adhesion molecules in human endometrium. *Am. J. Clin. Pathol.,* **93,** 183-189.
- Tabibzadeh, S. (1991) Induction of HLA-DR expression in endometrial epithelial cells by endometrial T cells: potential regulatory role of endometrial T cells in vivo. *J. Endocrinol. Metab.,* **73,** 1352-1359.
- Tabibzadeh, S. (1992) Patterns of expression of integrin molecules in human endometrium throughout the menstrual cycle. *Hum. Reprod.,* **7,** 876-882.
- Tabibzadeh, S., Kong, Q.F., Satyaswaroop, P.G., Zupi, E., Marconi, D., Romanini, C. and Kapur, S. (1994) Distinct regional and menstrual cycle dependent distribution of apoptosis in human endometrium. Potential regulatory role of T cells and TNFα. *Endocr. J.,* **2,** 87-95.

- Tabibzadeh, S. and Babaknia, A. (1995) The signals and molecular pathways involved in implantation, a symbiotic interaction between blastocyst and endometrium involving adhesion and tissue invasion. *Mol. Hum. Reprod,* **1,** 1579-1602.
- Tabibzadeh, S., Zupi, E., Babaknia, A., Liu, R., Marconi, D. and Romanini, C. (1995) Site and menstrual cycle-dependent expression of proteins of the tumour necrosis factor (TNF) receptor family and bcl-2 oncoprotein and phase-specific production of TNFα in human endometrium. *Hum. Reprod., **10,** 277-286.*
- Tawia, S.A., Beaton, L.A. and Rogers, P.A.W. (1993) Immunolocalisation of cellular adhesion molecules, inter-cellular adhesion molecule-1 (ICAM-1) and platelet endothelial cell adhesion molecule (PECAM) in human endometrium throughout the menstrual cycle. *Hum. Reprod.,* **8,** 175-181.
- Tredway, D.R., Mishell Jr., D.R. and Moyer, D.L. (1979) Correlation of endometrial dating with luteinizing hormone peak. *Am. J. Obstet. Gynecol.,* **117,** 1030-1033.
- Vassiliadou, N. and Bulmer, J.N. (1996) Quantitative analysis of T lymphocyte subsets in pregnant and non-pregnant human endometrium. *Biol. Reprod.,* **55,** 1017-1022.
- Vassiliadou, N. and Bulmer, J.N. (1998) Functional studies of human decidua in spontaneous early pregnancy loss: effect of soluble factors and purified CD56+ lymphocytes on killing NK- and LAK- sensitive targets.
- Vassiliadou, N., Searle, R.F. and Bulmer, J.N. (1999) Elevated expression of activation molecules by decidual lymphocytes in women suffering spontaneous early pregnancy loss. *Hum. Reprod.,* **14,** 1194-1200.

- Watson, A.J., Kidder, G.M. and Schultz, G.A. (1992) How to make a blastocyst. *Biochem. Cell. Biol.,* **70,** 849-855.
- Wegmann, T.G. Lin, H., Guilbert, L. and Mosmann, T.R. (1993) Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a T_H2 phenomenon? *Immunol. Today,* **14,** 353-356.
- Wentz, A.C., Kossoy, L.R. and Parker, R.A. (1990) The impact of luteal phase inadequacy in an infertile population. *Am. J. Obstet. Gynecol.,* **162,** 937-945.
- Wu, W-X., Brooks, J., Millar, M.R., Ledger, W.L., Glasier, A.F. and McNeilly, A.S. (1993) Immunolocalization of oestrogen and progesterone receptors in the human decidua in relation to prolactin production. *Hum. Reprod.,* **8,** 1129-1135.

APPENDICES

APPENDIX 1

Phenotype of CD56 positive decidual granulated lymphocytes.
(From Loke and King 1995)

Leucocyte common antigen
$CD45^{+}$

NK cell markers
$CD16^{-}$
$CD57^{-}$
$CD56^{bright}$

Early T cell markers
$CD2^{\pm}$
$CD7^{\pm}$
$CD38^{+}$

Mature T cell markers
$CD3^{-}$
$CD4^{-}$
$CD5^{-}$
$CD6^{-}$
$CD8^{-}$

Fibronectin receptors
$\alpha_4\beta_1^{++}$
$\alpha_5\beta_1^{++}$
$\alpha_4\beta_7^{++}$

Laminin receptors
$\alpha_6\beta_1^{-}$
$\alpha_1\beta_1^{++}$

HML-1 (mucosal lymphocyte marker)
$\alpha_E\beta_7$ $(CD103)^{+++}$

Activation markers
$*CD69^{+}$
$Kp43^{+}$
$*HLA\text{-}DR^{-}$
$CD45RA^{+}$

Cytokine receptors
$*IL\text{-}2R\beta^{+}$
$IL\text{-}2R\alpha(CD25)^{\pm}$
$c\text{-}kit^{-}$
$IL\text{-}7R^{-}$
$IL\text{-}6R^{-}$
$IFN\text{-}R^{-}$
$IL\text{-}1R^{-}$
$TFNR^{-}$
$GM\text{-}CSFR^{-}$

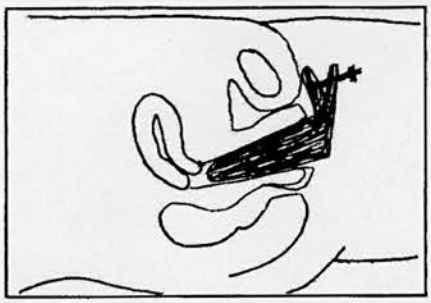
Ig superfamily
$ICAM\text{-}1^{++}$
NCAM-1 $(CD56)^{++++}$

β_2 integrins
CD11a $(LFA\text{-}1)^{+}$
$CD11b^{+}$
$CD11c^{++}$

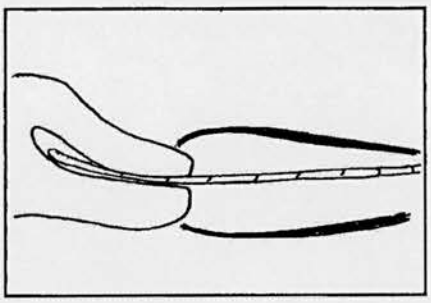
*Increased expression in some early spontaneous miscarriages (Vassiliadou et al. 1999).

APPENDIX 2

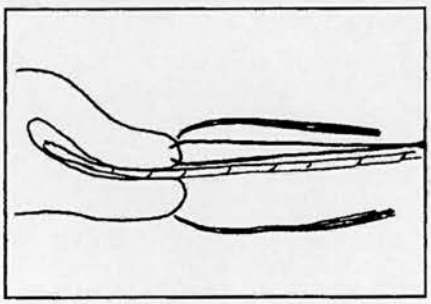
Endometrial sampling using Pipelle sampling device



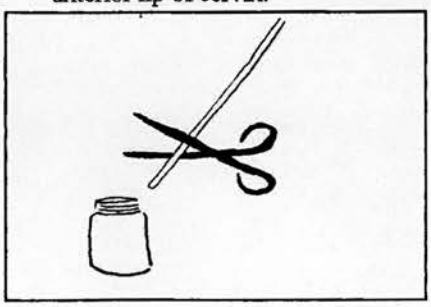
1. Cuscoes speculum inserted.



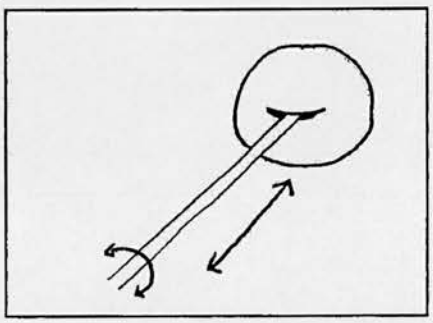
2a). Cervix visualised.
Pipelle inserted.



2b). Vulsellum applied if necessary to anterior lip of cervix.



4. Tip removed, plunger depressed to expel sample.



3. Pipelle rotated with in-out action to fill evacuated channel.

APPENDIX 3

CONSENT FOR RESEARCH

LYMPHOCYTE POPULATIONS IN HUMAN ENDOMETRIUM AND THEIR RELATION TO FERTILITY.

This study is intended to look at the types of white cells present in the lining of the womb. By looking at the nature and properties of these cells it is hoped that insight into previously unexplained infertility may be gained.

In order to do this samples of endometrium (from the lining of the womb) are required.

These samples are obtained by a small test taken in a similar way to a cervical smear. A small tube is passed through the cervix allowing a scraping of tissue to be obtained. Some crampy pain like a period pain may be experienced during this procedure but should settle immediately afterwards.

In order to be able to compare the samples from different women they need to be timed from mid-cycle, a urine test kit will be provided with instructions. Two samples are required per subject over two cycles. A blood test will also be required at the time of each sample to confirm your hormone concentration.

I have read and understood the above, and consent to take part in this study involving two timed "Pipelle" samples from the lining of the womb.

Signed _____

Print _____

Date _____

Witness_____

Print _____

Date _____

APPENDIX 4

EMBRYO CULTURE MEDIUM

Stock solutions

Sodium bicarbonate	25 mM
Sodium pyruvate	0.5 mM
Human serum albumin	50 mg/ml
Earle's balanced salt solution (EBSS)	284±2 mOsm

For 200ml stock culture medium

EBSS	20 ml
Sodium bicarbonate	20 ml
Water	Approx. 160 ml to give osmolality 284-286 mOsm

For 10ml 15% embryo culture medium

HSA	1.5ml
Culture medium	8.5ml
Sodium pyruvate	0.1ml

Final pH 7.4 in 5% CO_2.

Ethical Approval

Ethical approval for the above studies, excluding embryo supernatant pilot, was obtained from Joint Newcastle Health Authority / University of Newcastle-upon-Tyne Ethics Committee. Embryo supernatants were available under existing licence to HFEA.

Financial Support

This work was supported by grants from the Wellcome Trust and Newcastle and Associated Hospitals Special Trustees and also by Centre of Reproductive Medicine, Royal Victoria Infirmary, Newcastle-upon-Tyne.

Published /Presented Work

- Stewart, J.A., Murdoch, A.P., Bulmer, J.N. (1995) Steroid influences on endometrial leucocytes and their relation to fertility. *Hum. Reprod.* **10 (Abstract Book 1),** 26.

**Presented in poster form to the Joint Meeting of British Fertility Annual Conference, May 1995, Liverpool.

- Stewart, J.A., Bulmer, J.N., Murdoch, A.P., Searle, R.F. (1997) Are endometrial leucocyte populations affected by previous pregnancy? *J. Br. Fert. Soc.* **2,** 25A-26A.

**Presented in poster form to British Fertility Society Winter Workshop, December 1996, Dundee.

- Stewart, J.A., Bulmer, J.N., Murdoch, A.P. (1998) Endometrial leucocytes-expression of steroid hormone receptors. *J. Clin. Pathol.* **51,** 121-126.

- Jones, R.K., Searle, R.F., Stewart, J.A., Turner, S. and Bulmer, J.N. (1998) Apoptosis, bcl-2 and Ki67 expression in normal endometrium during the menstrual cycle, progesterone treated endometrium and early pregnancy. *Biol. Reprod.* **58,** 995-1002.

- Stewart, J.A., Bulmer, J.N. and Murdoch, A.P. (1999) Endometrial leucocytes and their relation to fertility. *Hum. Fertil.* **2.** 183.

**Presented as free communication to British Fertility Society Annual Conference 1999. Newcastle-upon-Tyne.

- Stewart, J.A., Searle, R.F., Bulmer, J.N., Murdoch, A.P. (2000) Endometrial steroid hormone receptor distribution in unexplained subfertility.

**Presented as free communication at Junior Clinician Prize Session, British Fertility Society Annual Conference, Edinburgh, 2000.

Papers / Presentations in Progress

- Endometrial steroid receptors in relation to fertility and parity

- Endometrial leucocyte populations in relation to fertility and parity

- Endometrial adhesion molecule distribution in relation to fertility and parity.

Acknowledgements

I would like to acknowledge Dr. Bulmer's research team in the Department of Pathology in particular Sarah Turner and Claire Gilfillan in their help and technical support in the laboratory and to recognise the benefit I have had from being part of that team.

I am grateful to the Team in Reproductive Medicine for their support and in particular to Jeanette Fenwick for collecting embryo supernatants.

To the women who supported this study by providing samples and sustaining interest over several months of involvement I wish good luck with my thanks.

I am grateful also to the valuable statistical support of Dr. Tony Hildreth who fitted some complex strategies amidst his otherwise very full schedule.

Above all however, I am wholeheartedly grateful to Doctors Judith Bulmer, Roger Searle and Alison Murdoch for their patient support throughout the execution of this work and since.

DECLARATION

I, Dr. Jane A. Stewart BSc (Hons.) (Edinburgh), MB,ChB (Edinburgh), MRCOG, declare that this thesis submitted for the Degree of MD to the University of Edinburgh was composed by me and that the work comprising it is my own. It has not been submitted in candidature for any other degree, diploma or professional qualification.

Jane A Stewart

24th July 2000